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Effects of substrate material on marine fouling community composition and ascidian larval settlement

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EFFECTS OF SUBSTRATE MATERIAL ON MARINE FOULING COMMUNITY COMPOSITION AND ASCIDIAN LARVAL SETTLEMENT

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ABSTRACT

EFFECTS OF SUBSTRATE MATERIAL ON MARINE FOULING COMMUNITY COMPOSITION AND ASCIDIAN SETTLEMENT

by

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University of New Hampshire, September 2015

Driven by the rising global population and shoreline development, man-made marine structures are becoming ubiquitous in coastal areas. These alterations may have enormous ecological consequences, as anthropogenic structures provide novel habitat for marine organisms and often host communities that differ from those on natural substrates. These community differences are frequently driven by non-native species, which can be more prevalent on man-made marine structures than on adjacent natural surfaces. Although multiple factors, including light intensity, surface orientation, predation exposure, and habitat type, are known to contribute to these patterns, relatively few studies have directly examined the influence of substrate material on fouling community development and non-native species abundance.

I conducted laboratory and field studies examining larval settlement preferences and fouling community composition on commonly occurring natural (granite) and man-made (concrete, high-density polyethylene, and PVC) marine materials. During laboratory trials, I quantified larval substrate preferences of two introduced species of ascidian, *Botrylloides*

violaceus and *Ciona intestinalis*. In the field, I sought to determine if observed differences in community composition on man-made and natural substrates resulted from differential organism settlement, or variation in post-settlement survival and growth, on different substrate materials.

Results indicate that the effects of substrate on fouling community composition are material-specific; assemblages on concrete differed significantly from those on all other surfaces. These differences appear to result from variation in both organism recruitment and survival on different materials. Notably, I found that material classification, as "man-made" or "natural," was not a reliable predictor of either species composition or non-native species abundance. Larval substrate preferences were also observed in *B. violaceus* and *C. intestinalis* during lab trials. However, these laboratory settlement patterns did not match those observed in the field, indicating the importance of real-world studies when seeking to answer more applied ecological questions. The effects of material on both settlement and community development most likely resulted from variation in chemical leaching or surface pH, as roughness did not seem to explain the observed differences among plate types.

Information gained through these studies may help us to understand how substrate features can contribute to differences in fouling community composition and species abundance, and may inform decisions regarding material usage in marine construction. These results also highlight the importance of settlement plate material choice in experimental marine biology research, as plate material may influence experimental conclusions.

GENERAL INTRODUCTION

Species introductions in terrestrial, aquatic, and marine ecosystems are increasing in frequency due to climate change, population growth, and increasing geographic interconnectivity (Ruiz et al., 2000). Coupled with habitat loss and environmental change, non-native species introductions have the potential to dramatically impact ecosystems worldwide. Introduced species can directly impact native communities by preying upon, outcompeting, or displacing, native organisms, and can decrease native biodiversity by altering food webs and changing physical ecosystem conditions (reviewed in Ruiz et al., 1997).

Marine systems are especially prone to species introductions due to shipping, recreational boating, and aquaculture industries, which foster high rates of geographic interconnectivity and organism transport (Carlton and Geller, 1993; Floerl and Inglis, 2005). Non-native organisms are frequently relocated through ballast water uptake and discharge and movement of fouled boat hulls, oil platforms, and aquaculture gear (Lambert, 2002; Rocha et al., 2009). These submerged man-made and natural substrates are colonized by sessile marine fouling organisms, including ascidians, mollusks, sponges, corals, bryozoans, hydroids, and bivalves. The geographic extent of anthropogenic organism relocations often greatly exceeds these species natural dispersal capabilities. Although only a small percentage of introduced species successfully establish in new habitats (Mack et al., 2000), non-native organisms have the potential to alter benthic community composition and dramatically impact native species (reviewed in Molnar et al., 2008 and Bulleri and Chapman, 2010).

Ascidians, commonly known as sea squirts or tunicates, are one of the most commonly introduced sessile marine species due to their high tolerance for fluctuations in temperature and salinity, rapid growth rates, and production of large numbers of larvae (Lambert, 2002; Dijkstra et al., 2008; Epelbaum et al., 2009). Ascidian larvae have very short planktonic lifespans, but many species are capable of overgrowing other benthic organisms and attaching to a wide variety of both naturally occurring and man-made substrates (Lambert, 2002; Simkanin et al., 2012). These organisms frequently encrust aquaculture cages, boat hulls, and other underwater equipment, promoting geographic transport of these organisms and necessitating costly and time consuming cleaning procedures (Callow and Callow, 2002).

In addition to posing problems for various marine industries, introduced ascidian species have altered benthic community composition in many areas, including the Gulf of Maine. The invasive colonial tunicate *Didemnum vexillum* has dramatically impacted benthic communities on Georges Bank, where it now occupies vast areas of the seafloor (Lengyel, 2009). Similar, though less extreme, changes have also been observed over the past three decades in coastal Gulf of Maine areas. Communities have shifted from a *Mytilus edulis*-dominated system to one primarily composed of introduced colonial ascidian species including *Botrylloides violaceus* and *Didemnum vexillum*, though it is not certain if the establishment of these species caused reductions in mussel densities (Dijkstra and Harris, 2009). Both *B. violaceus* and *D. vexillum* have infiltrated benthic communities on natural rock and cobble surfaces(Dijkstra et al., 2007a), however, many introduced ascidian species are not able to persist in these areas. Although introduced tunicates often colonize natural substrates, they rarely dominate communities on these surfaces (but see Valentine et al., 2007) and are often much more prevalent on man-made (artificial) structures including docks and pilings (Lambert, 2002; Simkanin et al., 2012).

In addition to ascidians, many other benthic taxa exhibit dramatic differences in abundance on natural and man-made substrates, often causing resultant variation in fouling community composition on these surfaces. Disparities in community structure and species composition may result from several factors, including light exposure, surface orientation, predation exposure, and habitat type. Communities on submerged vertical and lower horizontal surfaces frequently differ from those on upper horizontal surfaces (Harris and Irons, 1982; Glasby and Connell, 2001; Miller and Etter, 2008). Whereas vertical areas are frequently dominated by sessile invertebrates, upper horizontal substrates are more commonly colonized by macroalgae (Miller and Etter, 2008). These differences likely result from the interacting effects of differential light exposure and sedimentation (Miller and Etter, 2008). Predation exposure can also be a powerful structuring force in benthic communities, and assemblages often vary on surfaces that are protected from, and exposed to, predation (Otsuka and Dauer, 1982; Dumont et al., 2011), though this is not always the case (see Miller and Etter, 2008). Because many man-made structures are suspended above the sea floor, these constructions can act as predation refuges for organisms that would be readily consumed, and prevented from establishing, on natural benthic substrates. The effects of predator exposure have been observed in Chile, where the introduced singular ascidian, *Ciona intestinalis*, is unable to colonize natural substrates due to heavy predation by invertebrates and fish (Dumont et al., 2011).

In addition to shading, surface orientation, and predation exposure, substrate type has been shown to influence fouling community composition. Assemblages on floating docks differ from those on natural surfaces (Holloway and Connell, 2002), even when substrate material is held constant (cement, Glasby and Connell 2001). These differences have been attributed, at least in part, to the physical conditions associated with floating docks. Organisms on docks and other

floating structures are exposed to swash and remain at a constant, shallow, depth throughout the tidal cycle. Due to depth stratification of larvae, floating docks and rocky reefs may experience differential exposure to larval organisms, perhaps explaining some of the observed variation in community composition (Holloway and Connell, 2002). However, benthic communities on seawalls can differ from those on nearby rocky reefs, suggesting that factors other than distance from the sea floor, predator access, or surface orientation, can influence benthic communities (Bulleri, 2005). Bulleri (2005) proposed that community differences might result from variations in recruitment onto substrates due to surface characteristics including weathering, or topography.

Another factor that may contribute to observed differences in fouling on natural and man-made surfaces is substrate composition. However, while many studies have contrasted fouling organism settlement and community development on artificial and natural structures (ex. floating docks and rocky reefs), few have sought to isolate the effects of substrate material on these processes. Studies conducted in the intertidal (Anderson and Underwood, 1994) and subtidal (McGuinness, 1989; Brown, 2005; Tyrrell and Byers, 2007; Andersson et al., 2009; Vaz-Pinto et al., 2014) have found that substrate material can influence community development. These studies have compared settlement on plates composed of concrete, fiberglass, plywood, and aluminum (Anderson and Underwood, 1994), basalt, concrete, and fiberglass (Vaz-Pinto et al., 2014), treated wood, rubber, steel, and PVC (Brown, 2005), shell, marble, slate, wood, steel, styrofoam, PVC, and rubber (Tyrrell and Byers, 2007), and coral, wood, rock, concrete, and Perspex (McGuinness, 1989), as well as concrete and steel pilings (Andersson et al., 2009). Differences in community composition appear to result from species level variations. A study conducted by Andersson et al. (2009) in Sweden found that communities on steel pilings were dominated by *C. intestinalis,* barnacles (*Balanus improvisus*), and calcareous tubeworms (*Pomatoceros triqueter*), whereas the

primary community constituents on concrete pillars were the hydroid *Laomedea* sp. and two other ascidian species (*Ascidiella spp.* and *Corella parallelogramma*). The influence of substrate material was also observed during a study conducted in Panama, where a greater number of algal species colonized rock, concrete, and Perspex plates than coral or wood surfaces (McGuinness, 1989). Similarly, an intertidal study in Australia observed that species settled on concrete and plywood in greater abundances than on fiberglass or aluminum (Anderson and Underwood, 1994). Tyrrell and Byers (2007) also found differences in the development of fouling communities on different materials; the abundance of introduced ascidian species increased more rapidly on man-made substrates (aluminum, Styrofoam, PVC, and rubber) than natural substrates (shell, marble, slate, wood) (Tyrrell and Byers, 2007). However, though the above studies have documented the effects of substrate material on benthic organisms, it is unknown if community differences result from differential larval settlement or variation in post-settlement growth or survival on different materials. Similarly, a greater understanding of how material characteristics, including surface roughness, microtopography, and chemical composition, may influence larval behavior and settlement preferences is necessary.

Knowledge of how substrate material influences benthic communities is of great importance due to the increasing ubiquity of man-made structures in marine habitats (Bulleri and Chapman, 2010). Underwater structures composed of concrete, plastic, metal, and other man-made materials, may serve as focal points for invasion (Lambert and Lambert, 2003). Therefore, knowledge of how communities on artificial substrate differ from those on natural benthic surfaces could inform decisions regarding material usage in marine construction and benefit aquaculture and other maritime industries. This information could also be applied to the design of early detection or monitoring systems for invasive species. Previous research has found that some

species of invasive ascidian will not settle on certain materials, necessitating the use of multiple substrates for invasive species monitoring (Cangussu et al., 2010). Exploration of substrate preferences could potentially allow scientists to encourage settlement of target species, fostering earlier detection of newly introduced organisms.

A more complete understanding of how substrate material influences settlement and community development is also necessary because benthic studies often examine sessile marine organisms on experimental settlement plates or panels. Researchers have used a wide variety of substrates, including glass (Scheer, 1945; Knight-Jones and Stevenson, 1950), asbestos board (Vandermeulen and Dewreede, 1982), marine plywood (Glasby, 2000), fiberglass (Floerl et al., 2004), Plexiglass® (Dijkstra et al., 2007a, 2007b), unglazed ceramic (Agius, 2007), tumbled slate (Kim and Micheli, 2013), roughly surfaced granite slabs (Miller and Etter, 2008), concrete (Connell, 2000, 2001; Glasby and Connell, 2001), roughened white polystyrene (Ramadan et al., 2006), acrylonitrile butadiene styrene (ABS) plastic (Nelson and Craig, 2011), epoxy, polyester, and silanised epoxy resins (Thomason et al., 2002), and polyvinyl chloride (PVC) (Osman and Whitlatch, 1995a; Stachowicz et al., 2002; Blum et al., 2007; Osman and Whitlatch, 2007; Janiak et al., 2013; Simkanin et al., 2013) as experimental settlement panels. Given the noted effects of substrate identity on communtiy development, it is unclear if results obtained using certain panel materials are truly generalizable. Further exploration of this topic is necessary to determine how settlement plate material may influence experimental conclusions.

The following studies sought to determine if substrate material influences fouling community composition in the southwestern Gulf of Maine. Experiments focused on ascidians, as these creatures are common fouling organisms that are often introduced into areas outside their native ranges due to human actions (Lambert, 2002; Dijkstra et al., 2008; Epelbaum et al., 2009). I

conducted field and laboratory studies using four experimental substrates (concrete, granite tile, high density polyethylene (HDPE), and PVC) to 1) determine if fouling community composition is influenced by substrate material, 2) determine if any observed differences in fouling community composition resulted from differences in recruitment onto panels of different materials or arose due to differences in post-settlement survival and growth, 3) derermine if man-made materials, as compared to a natural substrate, favored the settlement or growth of non-native species, 4) determine if two commonly occurring species of ascidian (*Ciona intestinalis* and *Botrylloides violaceus*) exhibited larval settlement preferences in the lab, and 5) determine if settlement and percent cover of these two ascidian species on field-deployed panels aligned with settlement patterns observed in the lab.

Common Gulf of Maine Ascidians

The solitary yellow-green sea vase tunicate *C. intestinalis*, is a cosmopolitan species complex that is found on every continent except Antarctica. *C. intestinalis* spB was introduced to the Gulf of Maine before the 1940's (Miner, 1950). Though it can colonize natural substrates, and is sometimes found on the undersides of overhanging rocks or in protected caves, it is primarily found on anthropogenic structures in the Gulf of Maine (Larry Harris, pers.com.).

The colonial ascidian *Botrylloides violaceus* is likely native to Japan and was established in the Gulf of Maine by 1981 (Berman et al., 1992). Coloration in this species is highly variable and can range from peach, yellow, or bright orange, to red, burgundy, or deep purple. This species readily colonizes subtidal rocks and algae and is commonly found on pilings, floating docks, and other submerged man-made structures (Carver et al., 2006).

Botryllus schlosseri is a colonial tunicate that was observed in the Gulf of Maine before 1870 and is found on natural and man-made substrates (Gould et al., 1870; Carver et al., 2006). Recent genetic analysis indicates that at least one haplotype clade of this species is native to the Gulf of Maine (Yund et al., 2015). Although recent introductions of non-native haplotypes have been observed (Yund et al., 2015), *B. schlosseri* was classified as a native species in the following studies. Zooids of *B. schlosseri* are oriented in a star of flower arrangement around shared excurrent siphons, lending this species its common name of golden star tunicate. *B. schlosseri* is extremely variable in color, with zooid pigmentation often contrasting with that of the surrounding matrix.

Diplosoma listerianum, a colonial species from Europe (Mackenzie, 2012), was first observed in the Gulf of Maine, at the Isle of Shoals, in 1993 (Harris et al. 1998). This gray-brown organism forms thin and relatively fragile mats on both natural and man-made surfaces and is a common epibiont.

Didemnum vexillum is a rapidly growing yellowish or cream-colored colonial ascidian that first appeared in Portsmouth Harbor in 2001 (Bullard et al., 2007). *D. vexillum* can attach to and grow upon almost any surface, and commonly colonizes pilings and dock floats, subtidal rocks, gravel, cobbles, boulders, seaweeds, eelgrass, and other benthic organisms (Bullard et al., 2007; Simkanin et al., 2013).

The singular ascidian *Styela clava* was introduced to the Gulf of Maine before 1988 and is found on natural and man-made substrates (Carlton, 1989; Locke et al., 2007). This species is attached to the substrate by a short stalk and has a woody tunic that is readily colonized by epibionts.

The sea grape tunicates *Molgula citrina* and *Molgula manhattensis* are both solitary species that readily colonize man-made and natural surfaces. Both *M. citrina* and *M. manhattensis* are native to the Gulf of Maine and globular in shape (Pollock, 1998). Because these species are very similar in appearance, and internal structures are often obscured by accumulated sediment on the tunic, dissection is often required for positive identifications (Pollock, 1998). Due to the limitations of non-destructive field sampling, *Molgula* spp. was used to denote all Molgulid tunicates during the following studies.

CHAPTER 1: THE INFLUENCE OF SUBSTRATE MATERIAL ON ASCIDIAN LARVAL SETTLEMENT

Introduction

Most benthic marine organisms have a biphasic life cycle, including a pelagic larval phase and a demersal, sessile, or sedentary adult phase. Life history strategies vary widely between taxa, but pelagic larval stages often facilitate the dispersal of juvenile organisms. Larvae can spend minutes to months in the water column before metamorphosing and settling onto the seafloor and other surfaces. The site of this eventual settlement is of particular importance for sessile organisms, which are unable to change their location following substrate attachment and metamorphosis. Accordingly, initial settlement patterns play a large role in the structuring of benthic communities, though these assemblages are also influenced by post-settlement mortality, competition, and growth (Connell, 1985; Hunt and Scheibling, 1997; Fraschetti et al., 2002).

Given the influence of settlement site selection on organism survival and fitness, it is unsurprising that many sessile animals exhibit active settlement preferences (e.g., tube-building polychaetes *Hydroides diantus* and hydroid *Ectopleura crocea*, Mullineaux and Garland, 1993; colonial ascidian *Diplosoma similis,* Stoner, 1994; hydroid *E. crocea* and barnacle *Balanus* sp., Lemire and Bourget, 1996; barnacle *Balanus crenatus,* Miron et al., 1996). Numerous factors are known to influence sessile organism settlement, including light intensity (Thorson, 1964; Durante, 1991; Rius et al., 2010), proximity of conspecifics or prey organisms (reviewed in Burke, 1986;

Pawlik, 1992), surface microtopography (Wethey, 1986; Kerr et al., 1999; Lemire and Bourget, 1996) and substrate chemical composition (Kerr et al., 1999; Bavestrello et al., 2000).

Many invertebrates initiate metamorphosis in response to chemical cues from conspecifics (reviewed in Burke, 1986 and Pawlik, 1992). Gregarious settlement has been observed in many taxa, including ascidians (*Chelyosoma productum,* Young and Braithwaite, 1980; *Botrylloides violaceus,* Westerman et al., 2009), abalone (Slattery, 1992), oysters (Hidu, 1969), and barnacles (*Elminius modestus,* Knight-Jones and Stevenson, 1950), and may facilitate successful reproduction or act to diffuse individual predation risk. However, larval behaviors are highly species-specific, and numerous benthic organisms, including the ascidians *Diplosoma listerianum, Botryllus schlosseri,* and *Molgula* spp., do not exhibit aggregative settlement (Schmidt, 1982; Durante, 1991). Chemical cues from prey or host organisms may also trigger larval settlement (Pawlik, 1992).

In addition to biological factors, abiotic conditions including light exposure, and substrate characteristics including color, roughness, and chemical composition, can influence larval settlement patterns. Many invertebrates, including most ascidians, exhibit preferential settlement on non-illuminated surfaces (Durante, 1991; Rius et al., 2010). This adaptation may serve to encourage settlement in areas where competition with, and overgrowth by, macroalgae is less likley. Substrate microtopography, which can influence flow conditions, shear stress, and the availability of dissolved gasses and food particles, can also effect larval settlement (Vogel, 1996). Rougher surfaces, which have more turbulent boundary layer flows, are known to facilitate the settlement of certain organisms, including hydroids, barnacles (Mullineaux and Butman, 1991; Wright and Boxshall, 1999), bivalves (Bologna and Heck, 2000), and polychaetes (Hurlbut, 1991; Walters et al., 1999). However, many other species preferentially settle on smooth surfaces,

(ascidians, bryozoans, polychaetes, Osman and Whitlatch, 1995a, 1995b; barnacles, Lemire and Bourget, 1996). Additionally, although surface roughness impacts settlement of many species (reviewed in Howell and Behrends, 2006), this is not always the case (Hurlbut, 1991; Orlov, 1996). Other characteristics including substrate color and chemical composition are also known to impact settlement, though few studies have directly examined these topics (Satheesh and Wesley, 2010; Bavestrello et al., 2000, respectively)

The above factors contribute to observed differences in fouling assemblages on man-made and natural substrates. These surfaces frequently differ in surface orientation, light exposure, proximity to the sea floor or water surface, predation exposure, and substrate composition, all of which are known to influence community development. Though multiple studies have compared fouling community assemblages on different materials in the field (McGuinness, 1989; Anderson and Underwood, 1994; Tyrrell and Byers, 2007; Andersson et al., 2009; Vaz-Pinto et al., 2014), relatively few studies have directly examined larval settlement preferences for different materials (scyphozoa, Holst and Jarms, 2006; Hoover and Purcell, 2008). Laboratory experiments that examine larval substrate preferences in isolation are a valuable complement to field-based community studies, and can help identify whether initial settlement density and/or post-settlement mortality and growth rates are responsible for differences in community composition on natural and man-made structures.

To determine if larval settlement preferences may contribute to observed differences in ascidian abundance on natural and man made surfaces in the Gulf of Maine, I examined two commonly occurring species, the solitary *Ciona intestinalis* and the colonial *Botrylloides violaceus*. Larvae were exposed to settlement plates composed of concrete, granite tile, high-density polyethylene (HDPE), and polyvinyl chloride (PVC) during controlled laboratory trials. In

the Gulf of Maine, *B. violaceus* and *C. intestinalis* are non-native, and cryptogenic, respectively. Therefore, I hypothesized that larvae of both species would exhibit active substrate preferences during settlement trials and would settle more frequently on artificial substrates than on a pseudo-natural substrate (granite tile).

Methods

Study species

Both *Ciona intestinalis* and *Botrylloides violaceus* commonly occur on man-made structures, and also colonize natural substrates, in the southwestern Gulf of Maine. *C. intestinalis,* is a cosmopolitan species complex composed of at least three genetically distinct but morphologically similar species (Suzuki et al., 2005; Caputi et al., 2007). *C. intestinalis* spB was introduced to the Gulf of Maine before the 1940's (Miner, 1950) and has become a common and often dominant fouling community constituent on floating docks in sheltered coastal areas. Fertilization of *C. intestinalis* eggs occurs in the water column following gamete release during summer and fall months. The duration of embryonic development is highly dependent upon temperature and can range from 18 hrs at 18-20°C to 48 hrs at 12°C (Dybern, 1965; Bullard and Whitlatch, 2004). *C. intestinalis* larvae are very small (0.88-1.28mm in length) and nearly transparent with a larval phase ranging from 24 hours at high temperatures (18-20°C) to 5 days at low temperatures (10-12°C) (Dybern, 1965; Bullard and Whitlatch, 2004).

My second study species, *B. violaceus,* is a colonial ascidian that is likely native to Japan, and has been established in the Gulf of Maine since 1981 (Berman et al., 1992). *B. violaceus* is a common fouling organism and is found on both man-made and natural substrates including floating docks, pilings, subtidal rocks and algae, and *Mytilus edulis* shell (Carver et al., 2006). This

species produces very large (length <3 mm) and often brightly colored orange, red, pink, or purple tadpole larvae that are brooded within the colony and released throughout the summer and fall (Bullard and Whitlatch, 2004). The *B. violaceus* larval phase is very short, lasting from several minutes to hours after release from the parent colony (Lambert, 1990)

Specimen collection

B. violaceus larvae were obtained from mature colonies collected from floating docks composed of HDPE at Wentworth Marina in New Castle, NH, from shallow subtidal rock surfaces and algae at Odiorne Point State Park, Rye, NH, and from subtidal rocks and algae via scuba diving at Cape Neddick, York, ME. *C. intestinalis* gametes were obtained from individuals collected from HDPE floating docks at Hampton River Marina, Hampton Beach, NH and Salem Marina, Salem, MA, and from HDPE floating docks and concrete and steel underwater structures at the UNH Coastal Marine Lab Pier in Newcastle, NH.

Experimental substrates

Four materials were selected to represent natural and man-made substrates typical of Gulf of Maine coastal systems. Concrete (commercial grade Quickrete® quick-setting cement) and black high-density polyethylene (King StarBoard® marine building material, HDPE) were chosen as experimental substrates because these materials are extremely common in developed marine areas. Gray chemical-resistant type 1 PVC was included in this study because it is a commonly used settlement plate material in scientific studies examining marine fouling communities (Osman and Whitlatch, 1995a; Stachowicz et al., 2002; Blum et al., 2007; Osman and Whitlatch, 2007; Janiak et al., 2013; Simkanin et al., 2013). Granite substrates were included because this mineral is

the primary component of ledge and bedrock in the southwestern Gulf of Maine. Standardized natural rock samples could not be obtained, so the unpolished sides of dark gray granite tiles were used as pseudo-natural substrates.

Larval acquisition

B. violaceus

Colonies were collected in the afternoon and housed overnight in aerated 15°C seawater aquaria under fluorescent lighting on a 12hr:12hr light: dark cycle. Larval release began rapidly upon illumination the following morning and continued from approximately 8am to 3pm. Active larvae were removed from containers using a pipette and placed into a glass holding dish as they were released from parent colonies. Larvae were counted immediately before the start of trials, which began between 11:00am and 2:00pm.

C. intestinalis

Eggs and sperm were obtained via dissection of mature individuals and fertilized in vitro (procedure modified from Cirino and Brown, 2014). A longitudinal incision was made through the tunic of each individual *C. intestinalis* and eggs were removed from the oviduct using a pipette. Eggs from 6 to 10 individuals were hydrated in at least 6 dishes containing approximately 100 ml of seawater for 15-30 minutes before sperm addition. One drop of sperm was removed from 4 to 7 individuals and mixed with 100 ml of fresh seawater approximately 5 minutes before gamete mixing to promote maximum motility. A sample of the sperm mixture (~ 5ml) was added to each dish of eggs and embryogenesis was monitored until larvae were fully developed. Excess sperm was removed 1 hour after fertilization to minimize embryo mortality by removing several ml of

water from each dish and replenishing containers with fresh seawater. The required number of larvae was removed prior to the start of trials, which began between 1:00pm and 5:00pm.

Settlement preference

Four 10 x 10cm plates (one each of concrete, granite tile, HDPE, and PVC) were suspended with their lower surfaces approximately 2mm below the water surface in aquaria filled with 2000ml of seawater (Fig. 1). A short section of PVC pipe was attached to the upper surface of each plate with monofilament line. Plates were then suspended within aquaria by a strand of monofilament line that was threaded through the PVC pipe and attached to a section of plastic-coated wire mesh that rested on top of each aquarium. The position of each plate material was randomized for each larval settlement trial. At the beginning of each trial, 100 *B. violaceus* or 250 *C. intestinalis* were added to each aquarium. More *C. intestinalis* larvae were used for each trial due to the smaller size and longer duration of the larval phase in this species, compared to *B. violaceus*. Aquaria were left undisturbed at 15°C until larval settlement was complete (>90% settlement as determined during pilot trials, *B. violaceus:* ~24 hours, *C. intestinalis:* ~72hrs). Plates were then examined and all settled individuals on the lower surface of each plate were counted. Counting techniques varied between species due to differences in settled ascidian size and color. *B. violaceus* settlers were easily identifiable and were counted without magnification. *C. intestinalis* individuals were identified by examining plates under a dissecting microscope.

Settlement preference based on substrate age

To determine if larval settlement preferences changed with substrate age, experiments were conducted using two groups of settlement plates. One set of plates were aged in seawater for 1 to 3 weeks (young) before the start of trials, while another group of plates were aged for 5 to 10 weeks

(old) before the start of trials. Between 4 and 8 trials (1 trial = 1 aquarium) were performed simultaneously until at least 16 trials were completed for each species using both young and old plates. Trials were conducted when larvae and mature gametes of each species were most abundant and readily obtainable; *B. violaceus* trials were conducted between July 29 and September 18, 2014. *C. intestinalis* trials were conducted from October 10 to 24, 2014.

Statistical analysis

Counts of settled larvae on each substrate were analyzed to examine settlement preferences of *C. intestinalis* and *B. violaceus*. Count data from all trials, for each species and level of plate aging, were pooled for analysis. Because Chi square and G-tests of goodness-of-fit can yield erroneously low p-values when expected values are below 5 and total sample size is less than 1000, exact multinomial tests of goodness-of-fit were used. These analyses tested the null hypothesis that ascidian larvae were not exhibiting material-specific settlement preferences, assuming a 1:1:1:1 distribution of settled individuals among the four plate materials. Post-hoc binomial tests were used to compare the number of settled individuals on each substrate to that expected by chance. As individual binomial tests were used to examine settlement on each of the four substrate materials, the significance level was adjusted from $\alpha = 0.05$ to $\alpha = 0.0125$ for these analyses to account for multiple comparisons. To determine if substrate preferences were influenced by parent organism location, counts were pooled by collection location and analyzed as above. All tests were performed in R, using the Stats (R Core Team, 2015) and Xnomial packages (Engels, 2014).

Surface roughness

A Mitutoyo Surftest SJ-400 portable surface roughness tester was used to obtain quantitative measurements of experimental settlement plate characteristics. This device measures roughness by drawing a stylus with a 2 μm-wide diamond tip across a surface for 4mm and recording micro-scale changes in elevation. The SJ-400 generates both surface roughness profiles and standard roughness metrics, including R_a, which is the arithmetic mean of deviations in surface height from the average. A perfectly smooth surface with no imperfections (deviations from the mean surface height) would have an R_a of 0; increasing R_a values indicate greater roughness. To compare roughness among experimental substrates, six randomly placed measurements were taken on three plates of each material (concrete, granite, HDPE, and PVC). Three values were recorded from the central 4 x 4cm area of each plate, and three values were recorded from outside this area, to evaluate if roughness varied with proximity to plate edges. R_a values were analyzed using one-way ANOVAs using SPSS statistics version 22 software.

Results

Between 12% and 29% of ascidian larvae introduced into aquaria settled upon experimental plates (Table 1.1). Interestingly, while *B. violaceus* larvae settled more readily onto 1-3 week aged plates than 5-10 week aged plates, this pattern was reversed in *C. intestinalis*.

Both *C. intestinalis* and *B. violaceus* exhibited non-random settlement onto experimental substrates (exact multinomial tests, all $P > 0.05$). These preferences differed between species and plate aging treatments. When exposed to 1-3 week aged plates, *B. violaceus* settled more often on concrete panels, and less often on all other materials, than expected by chance (Figure 1.3, Table 1.2). These patterns shifted slightly during trials using 5-10 week aged substrates; settlement onto

both concrete and HDPE plates was greater than expected (Figure 1.3, Table 1.2). Unlike *B. violaceus*, C. *intestinalis* settlement patterns changed only slightly between plate ageing treatments. Significantly more individuals settled onto both "young" and "old" HDPE plates than expected if larvae were not exhibiting preferential settlement. Counts of individuals on all other materials were lower than projected, with the exception of 1-3 week aged concrete plates, which did not differ significantly from the expected value.

Parent organism collection location seemed to have little to no effect on offspring settlement preferences for both species. *B. violaceus* larvae from colonies collected at both Odiorne State Park and Wentworth Marina settled onto concrete panels more often than expected during 1-3 week aged plate trials (Figure 1.3, Table 1.3). However, when presented with 5-10 week aged plates, larvae from Odiorne State Park settled more frequently onto concrete panels, while individuals from Cape Neddick settled most frequently onto HDPE surfaces (Figure 1.3, Table 1.3).

Similarly, *C. intestinalis* settlement preferences differed with parent collection location during trials using 1-3 week aged plates, but not 5-10 week aged plates. During "young" plate trials, counts of settled larvae were greater than expected by chance on HDPE plates for offspring of individuals from both Hampton and Salem Marinas (Figure 1.4, Table 1.4). However, larvae obtained from the UNH Coastal Marine Lab settled onto concrete plates more often than expected, and the number of individuals on HDPE did not differ from random (Figure 1.4, Table 1.4). During 5-10 week aged plate trials, offspring of *C. intestinalis* collected from all sites (UNH Coastal Marine Lab, Hampton Marina, and Salem Marina) demonstrated preferential settlement onto HDPE plates (Figure 1.4, Table 1.4).

Surface roughness

High-magnification images (Figure 1.5) and quantitative roughness profiles (Figure 1.6) suggest that experimental plates differed dramatically in surface microtopography. Analysis of roughness values (Ra) obtained from each substrate material support this conclusion. Surface roughness values did not differ between outer and inner areas of settlement plates; therefore all roughness measurements (six per plate) were averaged to yield mean roughness values. HDPE was significantly rougher, and PVC was significantly smoother, than all other plate materials, with average roughness values of 15.89 ± 0.93 (mean ± SD), and 0.243 ± 0.073, respectively (Figure 1.7, ANOVA, df = 3, F = 51.217, $p<0.001$; Tukey $p<0.05$). Granite (4.6 ± 1.02) and concrete (7.86 ± 2.88) were of intermediate roughness, between PVC and HDPE, and did not differ from one another (Figure 1.7, Tukey p = 0.136).

Discussion

Both *B. violaceus* and *C. intestinalis* exhibited substrate-specific settlement preferences. Though preferences differed between species, larvae only settled more frequently than expected by chance on HDPE and concrete plates (Table 1.2, Figure 2.2). The duration of pre-trial plate submersion influenced *B. violaceus* preferences, but not *C. intestinalis* settlement patterns (Table 1.2, Figure 2.2).

Data obtained during these trials represent active settlement preferences, though only about 25% of larvae settled upon experimental substrates (Table 1.1). Care was taken to select active larvae for each settlement trial, but it is likely that many individuals did not travel through the water column after introduction into experimental aquaria. These organisms likely sank to the bottoms of aquaria, where they attached and underwent metamorphosis. Due to time constraints, it

was not possible to quantify larval settlement onto aquarium surfaces. However, both *B. violaceus* and *C. intestinalis* were observed to swim sporadically, and tended to collect upon the bottom of holding containers, prior to the start of trials. All experimental substrates were suspended just below the water surface, requiring larvae to actively swim to reach these settlement sites. It can be assumed that all individuals that settled upon experimental plates were motile and physically able to contact the four substrate materials, whereas organisms that settled upon aquarium bottoms may not have been capable of active swimming and therefore could not have made contact with, and exhibited settlement preferences between, experimental substrates.

Though settlement frequency varied by species, and was influenced by pre-trial plate soaking for *B. violaceus*, larval counts were greater than predicted by a random model on concrete and HDPE plates (Table 1.2, Figure 1.2). Surface characteristics can influence the settlement of many benthic species, and numerous organisms preferentially settle upon topographically complex surfaces (barnacles Mullineaux and Butman, 1991; Wright and Boxshall, 1999)**,** bivalves (Bologna and Heck, 2000), and polychaetes (Hurlbut, 1991; Walters et al., 1999). *B. violaceus,* and especially *C. intestinalis,* may prefer HDPE due to its greater roughness than the other experimental substrates. Boundary layer flows are more turbulent and less laminar on rougher surfaces (like HDPE) than on smooth surfaces (like PVC) (Vogel, 1996). Greater turbulence leads to increased gas exchange rates and more mixing of fluid above the substrate. These flow conditions might allow ascidians to more easily acquire food and dissolved gases when settled on rougher substrates than on very smooth materials. Larvae were not exposed to either turbulent or laminar boundary layer flows during this study, but it is possible that behavioral preferences for settlement on rough materials may be heritable, and therefore observed even in environments with minimal or no water movement.

In addition to exposing organisms to potentially favorable boundary layer flows, settlement on rougher surfaces might be adaptive by reducing the risk of organism detachment from the substrate. Depending on the mechanism of adhesion, certain organisms may be able to form stronger attachments to rougher surfaces than to very smooth surfaces (Howell and Behrends, 2006). Although relatively little is known about the specifics of ascidian adhesion (Edlund and Koehl, 1998; Pennati and Rothbächer, 2015), both *C. intestinalis* and *B. violaceus* attach to substrates via protein-based glues. Because measured attachment strengths of colonial and solitary ascidians are considerably lower than many other benthic organisms, including mussels and barnacles (Murray et al., 2012), it is possible that substrate characteristics that influence attachment strength may play an important role in settlement for these animals. Though ascidians are able to reattach to surfaces through the growth of new tissue (Edlund and Koehl, 1998), this process is relatively slow, metabolically costly, and may not be common in nature, especially for solitary species like *C. intestinalis*. The relatively deep pits and valleys on the surface of HDPE plates (Figures 1.5, 1.6) might allow for more secure adhesion than the other experimental materials, especially the very smooth PVC.

In addition to directly altering flow conditions and adhesion strength, substrate roughness is also known to influence biofilm formation (Kerr et al., 1999; Kerr and Cowling, 2003). As biofilms can enhance or limit settlement, it is possible that indirect effects of roughness could be contributing to observed settlement patterns (Wieczorek and Todd, 1997; Maki et al., 2000).

Surface complexity alone does not seem to explain settlement patterns in *B. violaceus.* This species demonstrated a preference for concrete plates during trials using both 1-3 week and 5-10 week aged plates. Though concrete plates, on average, were rougher than all other materials save HDPE, there was no significant difference in R_a between concrete and granite tiles (Figure 1.7).

Therefore, it is possible that the chemical composition of substrate materials contributed to observed settlement patterns. Though the precise chemical components of the materials used in this study are uncertain, the composition of the four experimental substrates varied widely. Substrate material is known to influence the composition of microfouling communities, which can in turn effect macrofouling organism settlement (Marszalek et al., 1979). Additionally, mineralogical components including crystalline silica (quartz) have been found to directly impact larval settlement preferences in some species (hydroids, Bavestrello et al., 2000). Though both granite and concrete contain crystalline silica, settlement on these two substrates differed, perhaps as a result of the rate of leaching of this and other chemicals into the water column. It is possible that other, unidentified, chemical components may have influenced settlement preferences in *B. violaceus* and *C. intestinalis*.

Plate submersion time also influenced larval settlement patterns. Both *C. intestinalis* and *B. violaceus* exhibited greater settlement on concrete in trials using 1-3 week aged plates than in trials using 5-10 week aged plates (Table 1.2, Figure 1.2). This apparent change in the attractiveness of concrete may have resulted from decreases in chemical leaching with increased pre-trial submersion time. All settlement plates were soaked in fresh seawater for a minimum of one week before use in trials. Concrete plates, which were made specifically for this study, were observed to release a residue that formed a film on the water surface during the first several days of plate immersion. This discharge decreased with time. It is possible that both study species, and especially *B. violaceus*, were attracted to some of the chemical components that were released from concrete plates. Changes in settlement preferences between plate aging treatments might reflect decreased leaching rates of these chemicals with increased pre-trial submersion time.

It is also possible that differences in biofilm composition between 1-3 week and 5-10 week aged plates might have influenced settlement preferences. Biofilm composition and thickness, both of which can influence larval settlement, change with submersion time (Wieczorek and Todd, 1997). For example, Wieczorek and Todd (1997) found that *C. intestinalis* settlement increases with greater biofilm age. Though *B. violaceus* has not been studied in this context, it is possible that differences in biofilms, brought on by surface roughness, material chemical components, or substrate immersion time, might contribute to observed substrate preferences. However, trials examining the role of biofilms on settlement did not support this conclusion. Five- to ten-week-aged settlement plates were soaked in alcohol, rinsed with deionized water, dried, and immersed in seawater immediately before the start of trials using *B. violaceus.* This treatment resulted in no apparent changes in settlement, as compared to non-alcohol-cleaned plates. Though replication of this treatment is too low to draw definitive conclusions, and this trial was not repeated with *C. intestinalis*, these observations suggest that changes in chemical leaching, rather than differences in biofilm composition, may better explain differences in settlement between 1-3 week and 5-10 week aged plates.

Larval settlement patterns varied slightly between parent organism collection locations. However, these differences do not seem indicative of inherited material-specific substrate preferences (Tables 1.3, 1.4, Figures 1.3, 1.4). Although parent substrate material varied between Wentworth Marina (dock floats and algae) and Odiorne State Park (rocks and algae), *B. violaceus* larvae from both locations only settled more than expected by chance on HDPE plates during 1-3 week aged trials (Table 1.3, Figure 1.3). Similarly, all *B. violaceus* larvae used in trials with 5-10 week aged plates were collected from rocks and algae, therefore, the minor differences in settlement between offspring of Cape Neddick and Odiorne State Park individuals did not result

from inherited substrate preferences. *C. intestinalis* were only collected from dock floats (Coastal Marine Lab, Hampton Marina, Salem Marina) and a submerged metal wave-dampening wall (Coastal Marine Lab). Interestingly, offspring of individuals collected at the Coastal Marine Lab behaved differently than offspring from Salem or Wentworth Marinas (Table 1.4, Figure 1.4). Progeny of Coastal Marine Lab organisms preferentially settled on concrete plates, rather than HDPE plates, during trials using 1-3 week aged materials (Table 1.4, Figure 1.4). However, offspring of Coastal Marine Lab organisms did not exhibit unusual settlement patterns when exposed to 5-10 week aged plates (Table 1.4, Figure 1.4). Though it doesn't appear that parent organism substrate material systematically influenced either *B. violaceus* or *C. intestinalis* settlement preferences, this study did not initially seek to examine inherited substrate preferences, necessitating further examination of this topic.

Understanding the influence of substrate material on fouling organism settlement has important implications for experimental marine ecology. Relatively few settlement and benthic community studies have incorporated multiple substrate materials, and studies that utilize only one substrate type rarely offer a justification for this choice. As substrate material can influence settlement, future studies should seek to adequately justify material choice based upon experimental goals, or utilize multiple materials. These findings also highlight the importance of future research into substrate effects in the field. If the settlement patterns observed in this study are representative of field trends, it is possible that the use of concrete and HDPE for marine construction could encourage ascidian settlement. These two materials are widely used in marine systems, and could potentially foster the spread of non-native ascidians by providing preferred settlement sites.

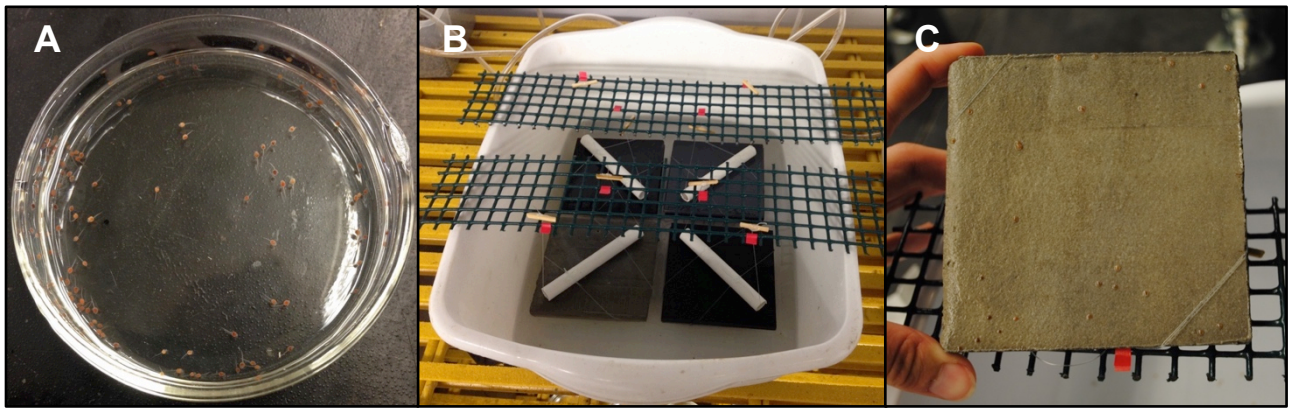

Figure 1.1. Larval settlement experimental setup. A) *B. violaceus* larvae in holding dish prior to start of trial. B) Larval preference experiment setup composed of four plates of different materials (concrete, granite, HDPE, PVC) with lower surfaces submerged. C) Concrete plate after ~24 hours of submersion with recently settled *B. violaceus*.

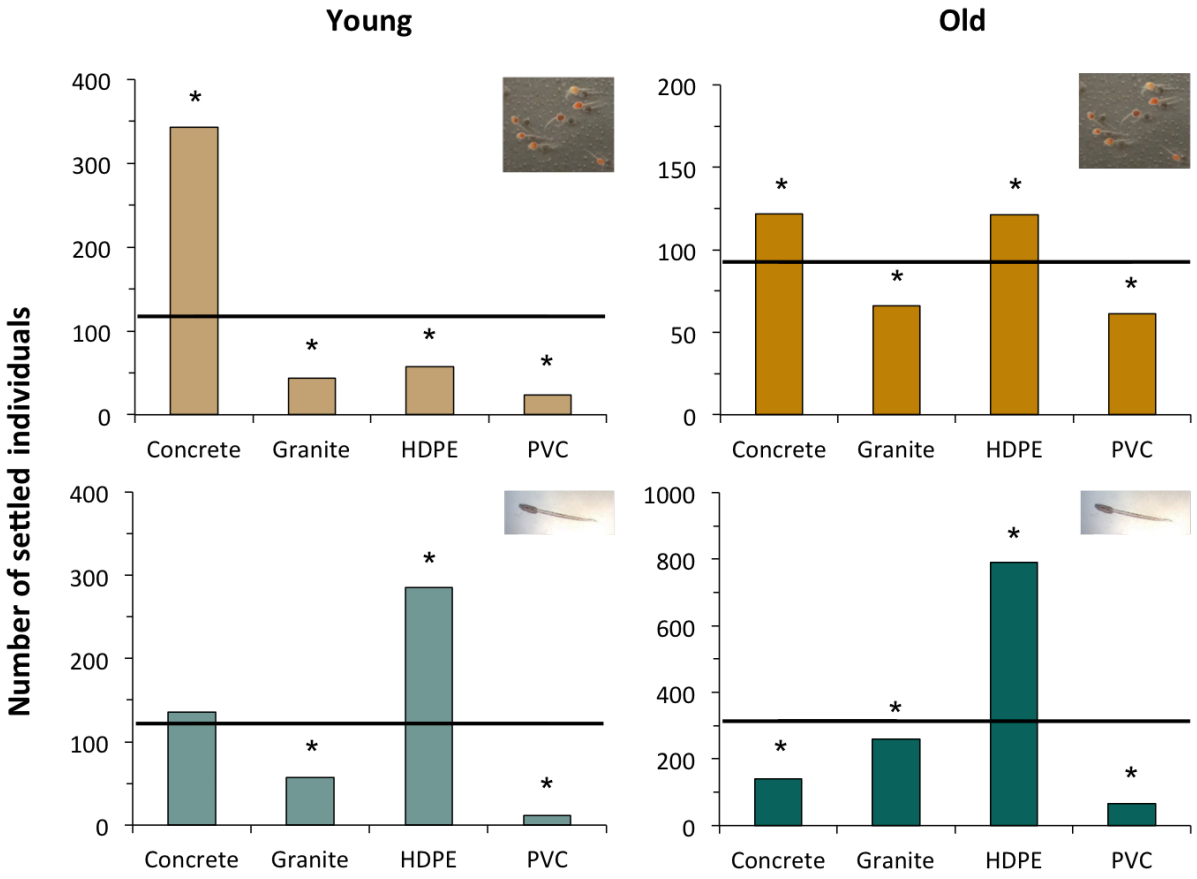

Figure 1.2. Total counts of *B. violaceus* (top) and *C. intestinalis* (bottom) individuals settled on each of four substrate materials during single-species settlement trials. Plates were aged for 1-3 weeks (young, $N_{C.}$=16, $N_{B.}$=16) or 5-10 weeks (old, $N_{C.}$=20, $N_{B.}$=19) prior to the beginning of trials. Horizontal lines denote counts of settled individuals expected if larvae exhibited no settlement preferences. Asterisks indicate values that differ significantly from expected counts (exact binomial tests, see Table 1.2).

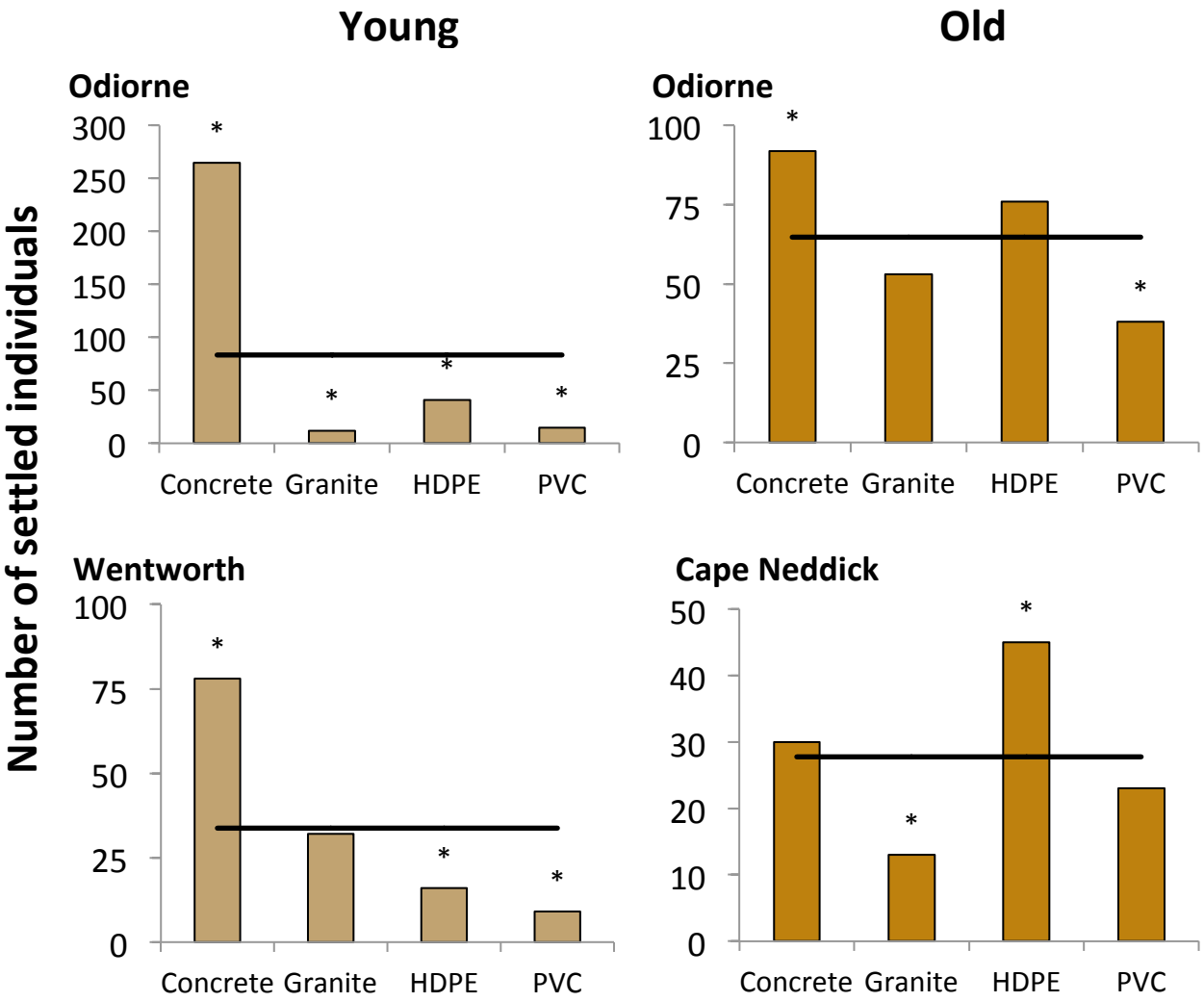

Figure 1.3. Settlement preferences of *B. violaceus* larvae obtained from parent colonies collected at different locations. Larvae were exposed to plates of four materials that were aged for 1-3 weeks (young) or 5-10 weeks (old) prior to the beginning of trials. Horizontal lines denote counts of settled individuals expected if larvae exhibited no settlement preferences. Asterisks indicate values that differ significantly from expected counts (exact binomial tests, see Table 1.3). Note variation in y-axis scale.

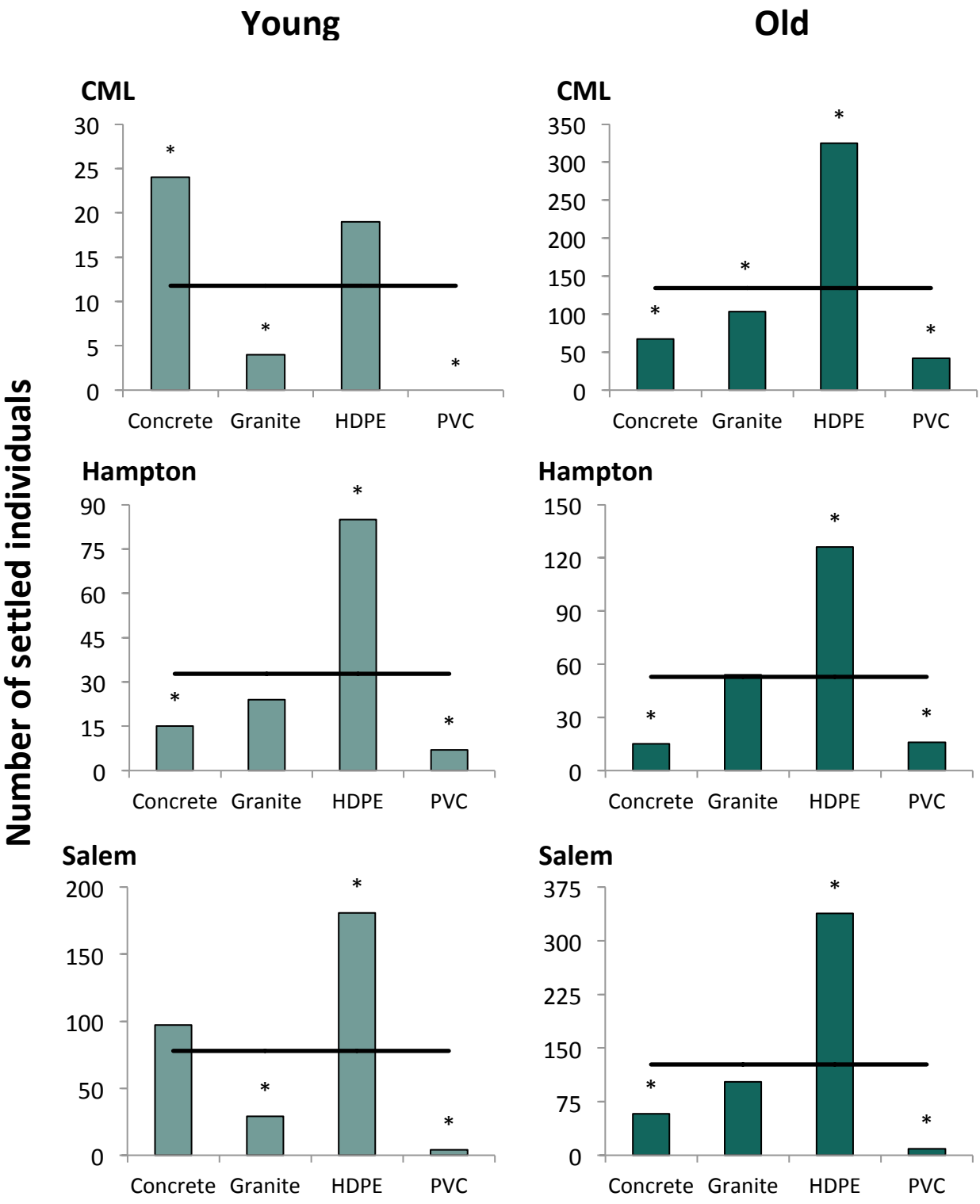

Figure 1.4. Settlement preferences of *C. intestinalis* larvae obtained from parent organisms collected at different locations. Larvae were exposed to plates of four materials that were aged for 1-3 weeks (young) or 5-10 weeks (old) prior to the beginning of trials. Horizontal lines denote counts of settled individuals expected if larvae exhibited no settlement preferences. Asterisks indicate values that differ significantly from expected counts (exact binomial tests, see Table 1.4). Note variation in y-axis scale.

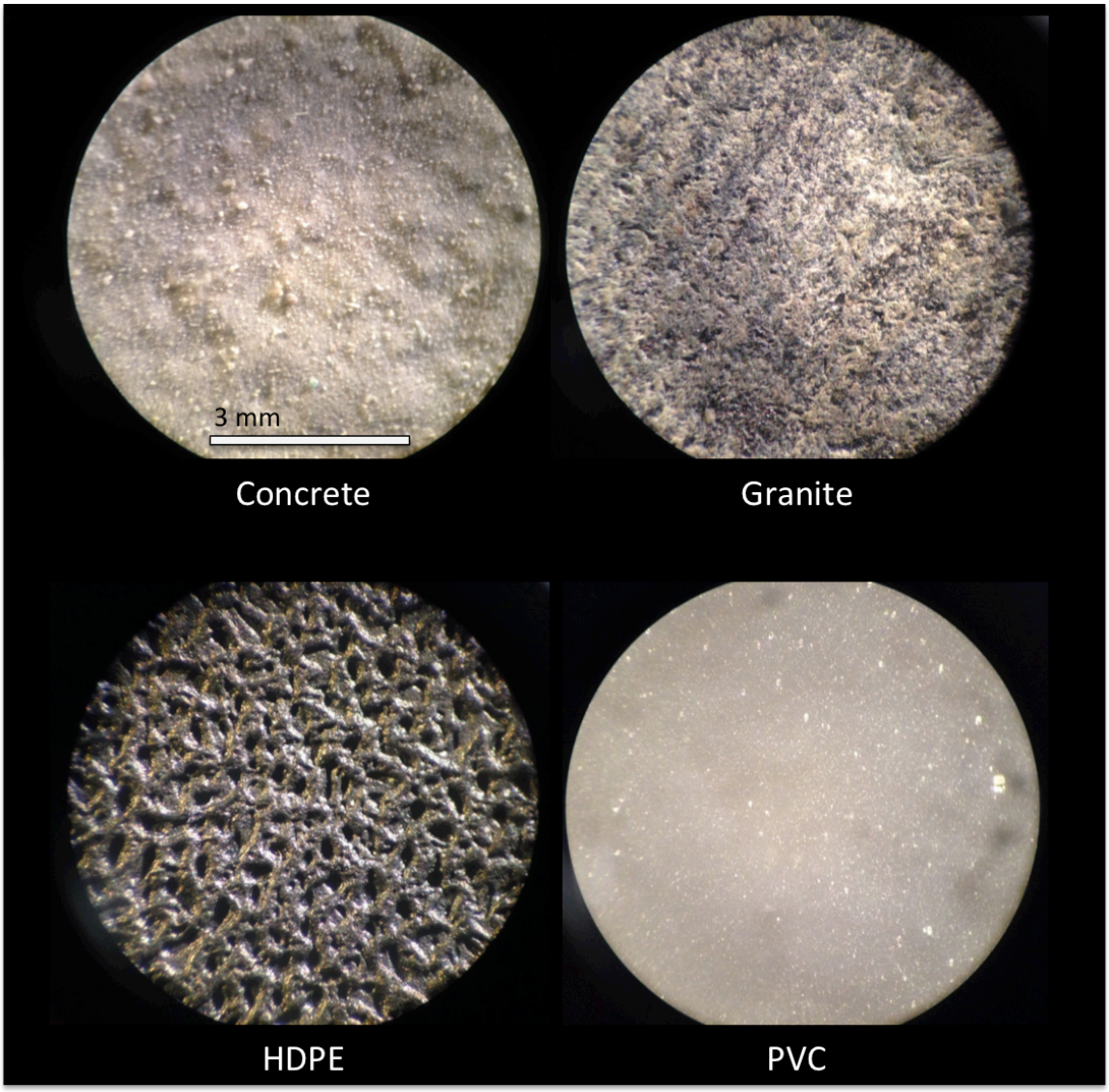

Figure 1.5. Representative photographs of concrete, granite, HDPE, and PVC settlement plate surfaces under magnification. Scale bar indicates 3mm.

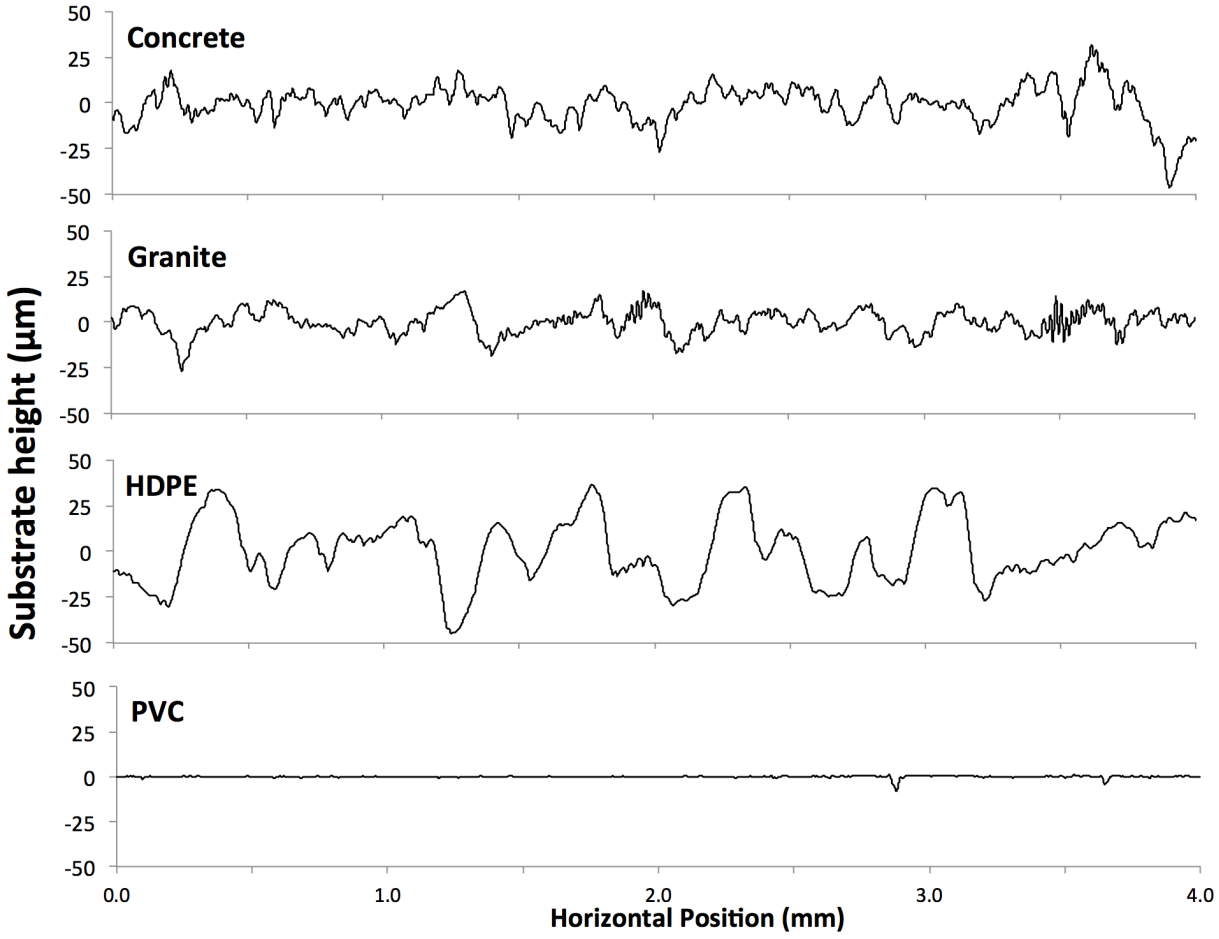

Figure 1.6. Representative surface roughness profiles of concrete, granite, HDPE, and PVC settlement plates. R_a for each sample: concrete= 7.25, granite= 4.25, HDPE = 16.06, PVC = 0.25.

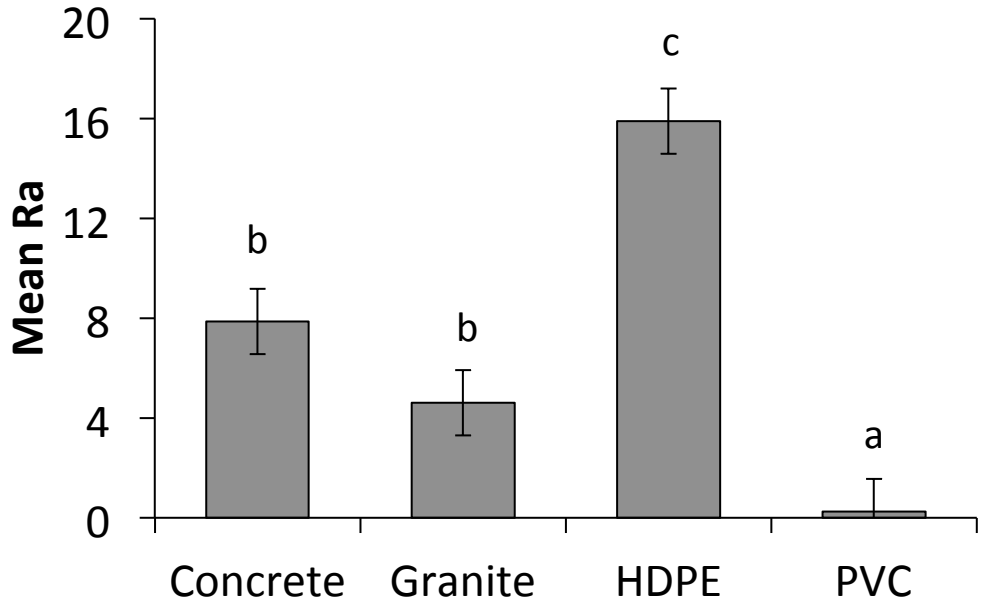

Figure 1.7. Mean surface roughness of experimental plates measured using a portable surface roughness gauge. Lower R_a values indicate smoother surfaces and higher values indicate rougher surfaces. Error bars denote ±1 SE, n=3 for each substrate. Shared letters indicate roughness averages that did not differ significantly.

Table 1.1. Percent of total ascidian larvae introduced into aquaria that settled upon experimental plates (concrete, granite, HDPE, PVC) during all trials.

Species	Plate age[a]	n	# total larvae	# larvae on plates	% settled on plates
B. violaceus	young	16	1600	468	29.25
	old	19	1900	370	19.47
C. intestinalis	young	16	4000	489	12.23
	old	20	5000	1256	25.12

[a] Duration of plate submersion in seawater before beginning of trials; young:1-3 weeks, old: 5-10 weeks.

Table 1.2. Results of exact binomial tests of goodness-of-fit comparing observed counts of ascidians settled on experimental substrates with expected counts assuming a null model of no settlement preferences. ns: p>0.0125, **p<0.01; ***p<0.001.

Species	Plate age[a]	N trials	substrate[b]	observed #	expected #	p
B. violaceus	young	16	C	343	117	***
			G	44	117	***
			H	57	117	***
			P	24	117	***
	old	19	C	122	92.5	***
			G	66	92.5	**
			H	121	92.5	***
			P	61	92.5	***
C. intestinalis	young	16	C	136	122.25	ns
			G	57	122.25	***
			H	285	122.25	***
			P	11	122.25	***
	old	20	C	140	314	***
			G	260	314	***
			H	789	314	***
			P	67	314	***

[a] Duration of plate submersion in seawater before beginning of trials; young:1-3 weeks, old: 5-10 weeks.
[b] C = concrete, G = granite, H = HDPE, P = PVC.

Table 1.3. Results of exact binomial tests of goodness-of-fit examining settlement of *B. violaceus* larvae obtained from parent organisms collected at different sites. Tests compared observed counts of ascidians settled on experimental substrates with expected counts assuming a null model of no settlement preferences. ns: p>0.0125, **p<0.01; ***p<0.001.

Species	Plate age[a]	Parent location	Parent substrate[b]	n trials	Substrate[c]	# Observed	# Expected	p
B. violaceus	young	Odiorne	R, A	12	C	265	83.25	***
					G	12	83.25	***
					H	41	83.25	***
					P	15	83.25	***
		Wentworth marina	D	4	C	78	33.75	***
					G	32	33.75	ns
					H	16	33.75	***
					P	9	33.75	***
	old	Odiorne	R, A	11	C	92	64.75	***
					G	53	64.75	ns
					H	76	64.75	ns
					P	38	64.75	***
		Cape Neddick	R, A	8	C	30	27.75	ns
					G	13	27.75	***
					H	45	27.75	***
					P	23	27.75	ns

[a] Duration of plate submersion in seawater before beginning of trials; young:1-3 weeks, old: 5-10 weeks.
[b] A = algae, D = floating dock, R = rock
[c] C = concrete, G = granite, H = HDPE, P = PVC

Table 1.4. Results of exact binomial tests of goodness-of-fit examining settlement of *C. intestinalis* larvae obtained from parent organisms collected at different sites. Tests compared observed counts of ascidians settled on experimental substrates with expected counts assuming a null model of no settlement preferences. ns: p>0.0125, **p<0.01; ***p<0.001.

Species	Plate age[a]	Parent location	Parent substrate[b]	n trials	Substrate[c]	# Observed	# Expected	p
C. intestinalis	young	Coastal Marine Lab	D	8	C	24	11.75	***
					G	4	11.75	**
					H	19	11.75	ns
					P	0	11.75	***
		Hampton Marina	D	4	C	15	32.75	***
					G	24	32.75	ns
					H	85	32.75	***
					P	7	32.75	***
		Salem Marina	D	8	C	97	77.75	ns
					G	29	77.75	***
					H	181	77.75	***
					P	4	77.75	***
	old	Coastal Marine Lab	D	4	C	67	134.25	***
					G	103	134.25	**
					H	325	134.25	***
					P	42	134.25	***
		Hampton Marina	D	4	C	15	52.75	***
					G	54	52.75	ns
					H	126	52.75	***
					P	16	52.75	***
		Salem Marina	D	8	C	58	127	***
					G	103	127	ns
					H	338	127	***
					P	9	127	***

[a] Duration of plate submersion in seawater before beginning of trials; young:1-3 weeks, old: 5-10 weeks.
[b] A = algae, D = floating dock, R = rock
[c] C = concrete, G = granite, H = HDPE, P = PVC

CHAPTER 2: THE EFFECTS OF SUBSTRATE MATERIAL ON MARINE FOULING COMMUNITY DEVELOPMENT

Introduction

Urbanization and development are dramatically increasing the amount of man-made structures in coastal waters (Bulleri and Chapman, 2010). Driven by the rising global population, these alterations may have enormous consequences for ecosystems around the world (reviewed in Bulleri and Chapman, 2010). Anthropogenic structures provide novel habitat for marine organisms, and often host communities that differ from those on natural substrates (Glasby, 1999; Connell, 2000). These community differences are frequently driven by non-native species, which are often more prevalent on man-made marine structures than on natural surfaces (Lambert and Lambert, 2003; Simkanin et al., 2012). Anthropogenic structures are at high risk of colonization by non-indigenous species, as these constructions are often located in high-traffic areas, and exposed to direct influxes of non-native propagules via aquaculture, shipping, and recreational boating activities (Carlton and Geller, 1993; Floerl and Inglis, 2005). Once established, populations of non-native organisms on docks, pilings, oil platforms, breakwaters, and other structures provide local larval sources and facilitate further range expansions via step-wise transport (Apte et al., 2000; Floerl et al., 2009). Although non-native propagule exposure certainly helps explain the high prevalence of non-native organisms on man-made substrates, many other factors likely contribute to observed differences between communities on anthropogenic and natural surfaces. As the

construction of anthropogenic marine structures continues to increase, it is vital that we understand what characteristics facilitate non-native species establishment in order to limit the spread of non-native organisms.

Several characteristics, which often vary systematically between man-made structures and natural substrates, are known to influence benthic community composition. These biotic and abiotic factors include substrate orientation and light exposure (Vandermeulen and Dewreede, 1982; Glasby and Connell, 2001; Thomason et al., 2002; Miller and Etter, 2008), proximity to the seafloor and water surface (Glasby and Connell, 2001; Holloway and Connell, 2002), and predation exposure (Otsuka and Dauer, 1982; Dumont et al., 2011). Anthropogenic structures may provide refuges from benthic predators and often present vertical or shaded lower horizontal surfaces for settlement, whereas natural substrates are usually more deeply submerged and accessible to predators. Certain habitat characteristics, including predation exposure, have been directly observed to limit non-native species abundance. For example, although the singular tunicate *Ciona intestinalis* is capable of recruiting on both natural benthic substrates and man-made materials in it's introduced range, this species is frequently unable to persist on natural surfaces due to heavy predation by invertebrate and fish predators (Dumont et al., 2011). However, organism responses to the above factors seem to be species-specific; unlike native invertebrates, which are more abundant on shaded surfaces, the non-native colonial ascidians *Didemnum vexillum* and *Botrylloides violaceus* are unaffected by light exposure (Miller and Etter, 2008).

Although differences in substrate orientation, predation exposure, and depth undoubtedly explain much of the noted variation in community composition and non-native species abundance on natural and man-made surfaces, the possible influence of substrate material on fouling community development remains understudied. Various factors known to influence marine

organism settlement, including substrate microtopography (Mullineaux and Garland, 1993; Lemire and Bourget, 1996) and chemical composition (Bavestrello et al., 2000), vary between man-made and natural materials, and may help to explain differences in community composition on these substrates. Previous studies have compared benthic communities on various natural and artificial materials in the intertidal (Anderson and Underwood, 1994) and subtidal (McGuinness, 1989; Brown, 2005; Tyrrell and Byers, 2007; Andersson et al., 2009; Vaz-Pinto et al., 2014). Many of these studies have found differences in fouling community composition or abundance of individual species on settlement plates or pilings composed of different materials. However, few studies have sought to isolate the effects of substrate material on both non-native species abundance and fouling community composition (but see Vaz-Pinto et al., 2014). Additionally, no previous study has sought to identify whether observed dissimilarities resulted from differences in larval settlement, or variation in post-settlement mortality and growth, on different materials.

In addition to identifying factors that may contribute to non-native species establishment and habitat dominance, a more thorough examination of how substrate material influences settlement and community development is necessary due to the extensive use of settlement plates in experimental marine biology. Researchers have utilized a wide variety of substrates, including glass (Scheer, 1945; Knight-Jones and Stevenson, 1950), asbestos board (Vandermeulen and Dewreede, 1982), marine plywood (Glasby, 2000), fiberglass (Floerl et al., 2004), Plexiglass® (Dijkstra et al., 2007a, 2007b), unglazed ceramic (Agius, 2007), tumbled slate (Kim and Micheli, 2013), roughly surfaced granite slabs (Miller and Etter, 2008), concrete (Connell, 2000, 2001; Glasby and Connell, 2001), roughened white polystyrene (Ramadan et al., 2006), acrylonitrile butadiene styrene (ABS) plastic (Nelson and Craig, 2011), epoxy, polyester, and silanised epoxy resins (Thomason et al., 2002), and polyvinyl chloride (PVC) (Osman and Whitlatch, 1995a;

Stachowicz et al., 2002; Blum et al., 2007; Osman and Whitlatch, 2007; Janiak et al., 2013; Simkanin et al., 2013) as experimental settlement panels. Given the possible effects of microtopography, chemical composition, and other substrate characteristics on larval settlement and subsequent development of fouling communities, it is unclear if results obtained using certain panel materials are truly generalizable.

I conducted a field study in the southwestern Gulf of Maine to determine if fouling community composition is influenced by substrate material, and to identify if differnces arose due to variations in settlement or post-settlement mortality and growth. I monitored bi-weekly settlement and observed community development throughout the summer and fall months on plates composed of concrete, granite tile, high-density polyethylene (HDPE), and PVC. I hypothesized that community composition would differ between substrate materials, with greatest differences between the pseudo-natural granite tiles and all other, man-made, substrates, and between HDPE and PVC (the most and least topographically complex surfaces – see Chapter 1). I expected to observe greater abundances of non-indigenous species on man-made plates (concrete, HDPE, and PVC) than on the pseudo-natural substrate (granite), a pattern that was observed previously in the Gulf of Maine by Tyrell and Byers (2007). I also predicted that abundance of the non-native tunicates *Botrylloides violaceus* and *Ciona intestinalis* would be greatest on concrete and HDPE plates, respectively, as larval settlement experiments conducted in the laboratory indicated preferences for these materials (see Chapter 1).

Methods

Experimental setup and sampling protocol

Twelve sets of four 10 x 10cm settlement plates (one each of concrete, granite tile, HDPE, and PVC) were suspended horizontally from a floating structure alongside a dock at the University of New Hampshire Coastal Marine Laboratory in New Castle, NH (Fig. 1, Fig. 2). Four plates (one of each experimental substrate) were attached to a 29 x 29 cm square of plastic-covered wire mesh using plastic coated wire. Four of these plate arrays were attached to each of three square 44 x 44 cm PVC frames using zip ties. All arrays were suspended from a 310 cm x 95 cm floating PVC frame with heavy-duty cord. Plates were randomly placed within each array.

The experimental apparatus was deployed on May 29, 2014. The lower surface of each plate was photographed and examined every two weeks through November 13, 2014. This submersion period allowed for the examination of settlement patterns and community development throughout the reproductive season for the majority of Gulf of Maine invertebrates. Half of the plate arrays (six randomly selected replicates of each substrate) were cleaned every two weeks to determine the effects of substrate type on fouling organism settlement, while half were left un-cleaned to examine community development.

Plate arrays were disconnected from the floating apparatus every two weeks and submerged in shallow containers for examination. The lower surface of each plate was photographed for percent cover analysis using a Nikon D3000 digital SLR camera with Nikor 18-55mm lens. Plates were then visually examined by at least two individuals to accurately assess presence/absence of all visible attached organisms, as certain small or translucent species were difficult to observe in photographs and percent coverage of organisms on bi-weekly cleaned plates

was low. Only the middle 8 x 8 cm of each plate surface was examined analyzed to minimize potential edge effects.

Image analyses

The central 8 x 8 cm of each plate was marked with a custom overlay using GIMP 2.8.10 software. Percent cover analysis of the marked area was performed by identifying organisms beneath 100 randomly placed points using Coral Point Count with Excel extensions software 4.1 utilizing a custom code file of Gulf of Maine fouling species (Kohler and Gill, 2006).

Images of bi-weekly cleaned plates were examined to determine the abundance of the hydroid *Ectopleura crocea*, the bivalve *Anomia simplex*, the bryozoan *Membranipora membranacea*, and four species of colonial ascidian (*Botrylloides violaceus, Botryllus schlosseri, Diplosoma listerianum,* and *Didemnum vexillum*). Counts could not be obtained for *C. intestinalis* or *Molgula* spp. due to the very small size and transparency of newly settled individuals of these species. Due to incredibly dense settlement of *Anomia simplex* at certain time points during the study, the abundance of this species was estimated by subsampling. Total abundances were extrapolated by counting individuals within 10 randomly selected 1 cm^2 areas of the plate surface.

Statistical analyses

Non-metric Multidimensional Scaling (NMDS) ordination was used to visualize divergence in community composition between substrate materials on both cleaned and un-cleaned plates. NMDS is a non-parametric distance-preserving ordination approach that reduces the complexity of multivariate data and is well suited for use on sparse data sets (Kruskal, 1964; Mather, 1976). All multivariate analyses were conducted in PC-ORD version 6 using Bray-Curtis (Sorensen) distance

measures (Bray and Curtis, 1957). Percent cover data were arcsine transformed and species abundances were generalized log transformed prior to analyses. All rare species (those occurring in <5% of samples) were excluded from analyses. Starting configurations were randomly assigned and PC-ORD Autopilot Slow and Thorough mode was used for all ordinations. The minimum number of dimensions necessary to accurately describe the data was determined by examining stress (ranging from 0-100) in relation to ordination dimensionality. Initial NMDS ordinations were produced with data from weeks 2-24 post-submersion for both un-cleaned plates (percent cover) and bi-weekly cleaned plates (species abundances and presence/absence data). To allow for finer scale examination of community differences, data from June-July (weeks 2-8), August-September (weeks 10-16), and October-November (weeks 18-24) were analyzed separately in subsequent analyses.

Multi-Response Permutation Procedure (MRPP), another non-parametric approach, was used to test for differences in community composition between substrate materials on un-cleaned and bi-weekly cleaned plates during June-July, August-September, and October-November. MRPP tests for differences between specified groups of sample units through an iterative randomization approach (Mielke and Berry, 2007). To adjust for 6 multiple comparisons between plate materials, the significance level for post-hoc pairwise tests was reduced to α=0.0083 (original α of 0.05 divided by 6).

Species most responsible for differences between groups were identified using Indicator Species Analysis (ISA) (Dufrêne and Legendre, 1997). ISA generates Indicator Values (IVs) for each species (the frequency of occurrence of a species in samples within a group multiplied by the relative abundance of a species in a group) that range form 0 to 100. A species present in all samples within a group and absent from all other groups (a perfect indicator) would have an IV of

100, whereas a species found in all samples in all groups would have an IV of 0. Species IVs were evaluated for significance using a Monte Carlo simulation with $\alpha=0.05$. ISAs were conducted on percent cover data from un-cleaned plates, and both presence/absence and abundance data from bi-weekly cleaned plates, in June-July, August-September, and October-November.

Two-way analyses of variance (ANOVAs) with substrate and time as factors were performed for all species present on at least 5% of plates. Variances remained heterogeneous (Levene's test, $p<0.05$) despite transformation, so untransformed abundance data and arcsin transformed percent cover data were used for all univariate analyses. Though ANOVA is relatively robust to heterogeneous variances (Box, 1953), analyses which failed to pass Levene's test were evaluated at a conservative significance level of 0.01.

To compare biofouling levels between substrates, total percent cover of all attached organisms and percent of bare plate surface were also analyzed with ANOVAs. To examine the potential influence of substrate material on the settlement and persistence of introduced species, percent cover (un-cleaned plates) and abundance (cleaned plates) data for all native, cryptogenic, and introduced species, were combined and analyzed with ANOVAs as detailed above (Table 2.1). I also calculated species richness, and Shannon and Simpson species diversity indices, for all plates. Richness data were obtained from visual examination of plates in the field, and percent cover (un-cleaned plates) and abundance (bi-weekly cleaned plates) data were used for diversity calculations. Rarefication was not necessary as species richness values were obtained using a census approach. All univariate analyses were preformed in IBM SPSS Statistics software version 22.

Results

Fouling assemblages on experimental plates changed dramatically throughout the 24 week observation period, but were primarily composed of ascidians (*Ciona intestinalis, Diplosoma listerianum, Botrylloides violaceus, Botryllus schlosseri, Didemnum vexillum, Molgula* spp.), bivalves (*Anomia simplex, Mytilus edulis, Hiatella arctica*), hydroids (*Ectopleura crocea, Obelia sp.*), sponges (*Halichondria panacea, Leucosolenia sp.*), and bryozoans (*Membranipora membranacea, Bugula turrita*) (Figure 2.3).

A 2-dimensional NMDS ordination (Figure 2.5) explained 90.7% of the variance in fouling community composition on un-cleaned plates from weeks 2 through 24 of the study (final stress= 13.58, axis 1 $R^2 = 0.438$, axis 2 $R^2 = 0.469$). As expected, time had a significant effect on community composition (MRPP, A=0.442, $p<0.00000001$). Interestingly, communities on concrete panels differed significantly from those on all other plate materials in both August-September (A=0.107, $p< 0.00000001$, C vs G, H, P all $p <0.00002$, N=5 each material) and October-November (A=0.100, $p< 0.00000001$, C vs G, H, P all $p<0.0000003$, N=5 each material), though not in June and July (MRPP: A=-0.011, p= 0.78, N=6 of each material). A total of 9 species were found to be significant indicators of different substrate materials (Table 1.2). Four of these organisms were indicators of concrete during both August-September and October-November, whereas all other substrates had no more than one significant indicator species throughout this time period. Indicators of concrete included *Halichondria panacea, Ciona intestinalis,* and *Botryllus schlosseri*, while *Membranipora membranacea* was indicative of PVC (Figure 2.6).

Univariate analyses of species percent coverage largely agreed with ISA results. A total of 9 species differed in percent cover between substrate materials (Figure 2.7, Table 2.3). Most of these organisms were present in greater or lower abundances on concrete than other materials, though *M. membranacea* occupied more space on PVC plates than on all other substrates (Table 2.7). Nearly all species identified as indicators also differed significantly between substrates, though *B. violaceus* and *M. citrina* (indicators of concrete and HDPE, respectively) coverage did not vary between materials (Table 2.7). Interestingly, though fouling community composition differed between concrete and all other substrates, this pattern was not reflected by the univariate community metrics of species diversity or richness (Table 2.6).

Total percent cover of fouling organisms differed between substrate materials. Concrete surfaces had significantly greater coverage of attached organisms, and less bare substrate, than all other materials (Figure 2.4, Table 2.3). Coverage of native, cryptogenic, and introduced species also differed between plate materials. Concrete plates had significantly greater coverage of native species, and lower coverage of introduced species, than other materials (Figure 2.8, Table 2.5). These patterns were driven primarily by *C. intestinalis*. This singular ascidian species was the lone cryptogenic organism observed on plates and covered more area on concrete than other materials (Figure 2.8, Table 2.5). When cryptogenic and introduced species were considered together, concrete plates had greater coverage of these organisms than both granite, and HDPE (Figure 2.8, Table 2.5).

In contrast to patterns observed on un-cleaned plates, community composition on bi-weekly cleaned plates did not differ between substrate materials in June-July, August-September, or October-November (all MRPPs; p/a: $p>0.1$; abundances: $p>0.2$, Figure 2.9). However, four species varied in abundance between substrates. *A. simplex,* and *M. membranacea*, indicator

species of un-cleaned concrete and PVC, respectively, were more abundant on bi-weekly cleaned plates composed of these materials than on other substrates (Figure 2.10, Table 2.4, Table 2.7). Abundances of *D. vexillum* and *D. listerianum* also varied between substrates (Figure 2.10, Table 2.4, Table 2.7). Introduced species (Table 2.1) were more abundant on granite and HDPE than on concrete (Figure 2.11, Table 2.7). Though concrete panels had higher abundances of native species than all other substrates, this pattern was driven solely by *A. simplex* (Figure 2.11, Table 2.5, Table 2.7). No cryptogenic species were observed on bi-weekly cleaned plates. As expected, community composition and species abundances changed with time (all MRPPs; p/a: $p<0.00000001$; abundances: $p<0.00000001$, Table 2.4).

Discussion

Contrary to my hypotheses, neither fouling community composition nor introduced species abundance was affected by substrate origin (natural or man-made). Although previous research has found that non-native species abundance is greater on artificial structures than on natural materials (Tyrrell and Byers, 2007; Dafforn et al., 2009), this was not the case in my study. Percent cover of non-native species on un-cleaned plates did not differ between the pseudo-natural granite substrate and any of the three artificial materials (concrete, HDPE, PVC, Table 2.5, Table 2.7, Figure 2.8). Furthermore, non-native species abundance on bi-weekly cleaned granite plates was greater than on either PVC or concrete plates (Table 2.5, Figure 2.11). Native species settlement (bi-weekly cleaned plates) and percent coverage (un-cleaned plates) also did not differ between natural and man-made materials (Table 2.5, Table 2.7, Figure 2.8, Figure 2.11). Similarly, neither species richness nor diversity differed between substrate types (Table 2.6), a finding that contradicts earlier observations that communities on artificial substrates have lower species richness than those on

natural materials (Chapman and Bulleri 2003, Firth et al. 2013). Fouling community composition on un-cleaned plates also did not differ between granite, HDPE, or PVC (Figure 2.5). As species and community-level responses appear to be material-specific, the classification of substrates as "natural" or man-made" is not useful (see Vaz-Pinto et al., 2014). It is likely that the noted differences in benthic community composition on natural and man-made marine structures arise from systematic variation in characteristics, like substrate orientation and light exposure (Vandermeulen and Dewreede, 1982; Glasby and Connell, 2001; Thomason et al., 2002; Miller and Etter, 2008), proximity to the seafloor or water surface (Glasby and Connell, 2001; Holloway and Connell), and predation exposure (Otsuka and Dauer, 1982; Dumont et al., 2011), rather than substrate material origin. Consequently, these characteristics should be given more attention than substrate material origin when considering the possible ecological impacts of coastal development.

Although fouling communities on granite plates did not differ from those on HDPE or PVC, community composition on un-cleaned concrete plates was significantly different than on all other substrate materials after two months of submersion. These community-scale differences were driven primarily by *A. simplex*, *B. schlosseri*, *C. intestinalis*, and *H. panicea*, which were all significant indicators of, and were more abundant on, concrete plates than granite, HDPE, or PVC plates (Table 2.2). Though three species were indicative of communities on HDPE (*D. vexillum*, *M. manhattensis*) or PVC plates (*M. membranacea*) (Table 2.2), community composition did not differ between these materials. Concrete plates also had significantly greater coverage of attached organisms, and less bare substrate, than all other materials (Table 2.3, Figure 2.4).

The observed differences between concrete and other plate materials likely resulted from differences in both initial settlement of organisms and post-settlement survival and growth rates on different substrates. Analysis of bi-weekly cleaned plates indicates that abundance patterns on un-

cleaned substrates seem to be driven by initial settlement for some species (*A. simplex*, *M. membranacea*) but not others (*B. violaceus*, *D. listerianum*, *D. vexillum*). However, many species that contributed to differences between communities on concrete and other plate materials were not present in high enough abundances on bi-weekly cleaned plates to allow for analysis (*C. intestinalis*, *B. schlosseri*, *H. panacea*, *M. edulis*). It appears that the extent to which initial settlement influences long-term abundance within fouling communities is species-specific.

Physical plate characteristics likely influenced both settlement and long-term survival on experimental substrates, resulting in the observed differences between concrete and all other materials. For example, surface microtopography, which can influence flow conditions, shear stress, and the availability of dissolved gasses and food particles (Vogel, 1996; Koehl, 2007), is known to influence the settlement of certain fouling organisms (reviewed in Howell and Behrends, 2006). Rougher surfaces, which have more turbulent boundary layer flows, are preferred by some hydroids, barnacles (Mullineaux and Butman, 1991; Wright and Boxshall, 1999), bivalves (Bologna and Heck, 2000), and polychaetes (Hurlbut, 1991; Walters et al., 1999). Conversely, many other species, including ascidians, bryozoans and polychaetes (Osman and Whitlatch, 1995a, 1995b) and barnacles (Lemire and Bourget, 1996) preferentially settle on smooth surfaces with more laminar flow. Surface roughness can also influence passive larval dispersal and settlement; turbulent fluid movement can lead to increased larval contact with rougher substrates (Eckman, 1990). However, roughness does not explain the differences in settlement and community composition observed in this study. Community composition did not differ between the roughest (HDPE) and smoothest (PVC) substrates (Figures 1.7, 2.5). Additionally, the roughness of concrete did not differ from that of granite (Figure 1.7), though these materials varied dramatically in terms of community composition and percent coverage of individual species (Table 2.3, Figure

2.5, Figure 2.7).

Although differences in roughness were not responsible for variation in community composition, it is possible that porosity or other structural characteristics may have influenced settlement. Though the porosity of experimental substrates was not determined, it is likely that concrete panels, which were cast specifically for this experiment, were more porous than granite tile and plastic plates (HDPE, PVC). In general, many larvae preferentially settle on porous materials (Pomerat and Weiss, 1946). It is possible that organisms may be able to form more secure attachments to more porous surfaces. This may help to explain why concrete plates had significantly greater coverage of attached organisms, and less bare substrate, than all other plate materials (Table 2.3, Figure 2.4). However, increased attachment strength may also be associated with substrate roughness.

In addition to substrate roughness and porosity, color may also influence settlement. Substrate color can impact both total fouling biomass (Satheesh and Wesley, 2010) and the recruitment of specific species (pearl oysters: Su et al., 2007; barnacles, sabellariid tube worms: Satheesh and Wesley, 2010). Su et al. (2007) and Satheesh and Wesley (2010) utilized plates of a variety of colors and observed greater recruitment and total biomass on darker (red and blue) substrates than on lighter surfaces (yellow, green, and white). In contrast to the trend observed by Satheesh and Wesley (2010), total coverage of fouling organisms was greater on the lightest substrate, concrete, than on all other, darker, materials in this study (Table 2.3, Figure 2.4). However, as the spectral sensitivity of most fouling organism larvae has not been determined, it is unclear if larvae were able to visually distinguish between concrete, granite, HDPE, and PVC substrates. The observed lack of preference for darker colored surfaces may also be attributed to the shaded position of experimental substrates, which may have minimized the spectral differences

between materials. Additionally, continuous growth and settlement of organisms onto un-cleaned plates changed the apparent color of large portions of each settlement plate, suggesting that substrate color was not the primary driver of fouling community composition during this study.

Although surface roughness, porosity, or color may explain some variation in fouling, it is likely that substrate chemical composition is most responsible for the observed differences among plate materials. Both man-made and naturally occurring chemicals can alter benthic organism settlement (Cerrano et al., 1999; Bavestrello et al., 2000; Callow and Callow, 2002; Yebra et al., 2004). Many antifouling paints and coatings function by creating low-friction surfaces and sloughing off settled organisms, however, antifouling biocides, like tributyltin (TBT) and other chemicals, actively deter larvae and kill organisms that settle upon treated surfaces (Callow and Callow, 2002; Yebra et al., 2004). Natural mineralogical components can also impact both larval settlement preferences and benthic community composition. Bavestrello et al. (2000) observed decreased settlement of the hydroid *Eudendrium glomeratum* on substrates containing high levels of quartz (crystalline silica). Quartz grains can also reduce organism settlement on marine soft-sediments (Cerrano et al., 1999), and benthic communities on quartzitic rock (granite, quartzite) have been shown to be less diverse than those on carbonic minerals (limestone, dolomite)(Bavestrello et al., 2000).

The diverse chemical components of the four substrates used in this study may have influenced fouling organism settlement, growth, competitive ability, and survival. Although both HDPE and PVC can release toxic gasses when exposed to direct sunlight, or burned, respectively, settlement plates were not subjected to these conditions during the study and both materials are non-toxic to humans under normal use (Material Safety Data Sheets: PVC and HDPE). None of the components of these materials have been observed to be toxic (PVC: PVC polymer, $CaCO_3$,

etc.), with the exception of titanium dioxide (TiO_2), which may be found in PVC (MSDS: PVC, TiO_2). Exposure to this substance has been observed to cause cellular and DNA damage in marine lugworms (*Arenicola marina*)(Galloway et al., 2010). However, the PVC used in this study contained only between 0 and 30% of $CaCO_3$ and TiO_2, which served as inert fillers. Leaching of TiO_2, a primary component of sunscreen, likely did not effect settlement during this study, as community composition did not differ between PVC, HDPE, or granite plates (Figure 2.5).

Unlike HDPE and PVC plates, both granite and concrete tiles contain quartz. Although concrete is composed of several other chemicals, including Portland cement, lime, alumina, limestone dust, and calcium sulfate, these materials are not known to be toxic in reasonable concentrations, though the effects of most of these substances on marine invertebrates have not been studied (MSDS: Quikrete, lime, alumina, calcium sulfate). Granite is composed primarily of quartz (70-72%) and feldspar, but can include many other minerals (MDSD: granite). Interestingly, community composition varied between concrete and granite plates, but not between granite, HDPE, and PVC plates (Figure 2.5). It is possible that these community differences may have resulted from differences in quartz exposure. Chemical leaching rates are positively related to porosity, therefore it is possible that larvae and settled organisms might have been exposed to higher levels of quartz (or other substances) on concrete plates (likely the most porous due to casting methods) than on other plate materials. Certain fouling species, like *C. intestinalis* and *A. simplex*, which were found in greater abundance on concrete than on other materials, may preferentially settle on quartz-rich substrates. Alternatively, organisms like *D. vexillum* or *D. listerianum,* that occurred less frequently on concrete than other materials, may be deterred from settlement or have lower survival rates when exposed to quartz. Future examination of the influence of quartz on Gulf of Maine fouling organisms is necessary to evaluate these hypotheses.

In addition to chemical composition, it is also possible that surface pH may help explain the observed differences in fouling assemblages between concrete and all other plate materials. pH affects many biological processes, including calcification, which is of great importance in marine environments (Orr et al., 2005; Kroeker et al., 2010). Exposure to acidified seawater decreases calcification rates in many species (Orr et al., 2005), with larval organisms often most sensitive to pH changes (Kurihara, 2008). Average seawater pH is currently 8.10 (Royal Society, 2005), though ocean acidification is predicted to reduce global pH by between 0.3-0.4 pH units by the end of the 21st century (Orr et al., 2005). Though pH was not measured in this study, Quikrete surface pH is 13 when freshly mixed (MSDS: Quikrete) and decreases with time through carbonation when exposed to CO_2 (Kakade, 2014). Carbonation can lower the surface pH of concrete to approximately 9, though this process can take over a year, depending on environmental conditions (Kakade, 2014). As concrete plates were approximately eight months old upon completion of this study, it is likely that surface pH on these panels was more alkaline than the surrounding seawater. If the alkalinity of concrete plates positively affected calcification rates, this might explain why certain calcifying species, like *A. simplex*, were present in greater abundances on concrete plates than on other plate materials. However, it is unclear if calcification in *A. simplex* and other organisms is affected by pH differences of the magnitude proposed here, necessitating further research.

Submersion time, in addition to substrate material, dramatically influenced both organism settlement on bi-weekly cleaned plates and community composition on un-cleaned plates. This was not surprising given natural successional processes and temporal variability in larval supply. Differences in community composition between plate materials were not immediately observed; communities on concrete panels did not differ from those on granite, HDPE, and PVC plates until

two months post-submersion (Figure 2.5). These patterns are not surprising, as total coverage of plates was low until several weeks post submersion. Previous studies have also found differences in community composition between vertically oriented intertidal settlement plates (Anderson and Underwood, 1994), subtidal steel and concrete pilings (Andersson et al., 2009), and steel, rubber, treated wood, and PVC plates (Brown, 2005) after one to three months of submersion. However, some evidence suggests that the effects of substrate material on community composition may be temporally limited. Anderson and Underwood (1994) observed community convergence on different substrates after 4-5 months of submersion, and although the effect of substrate was still significant after 12 months of submersion, Brown (2005) noted that community similarity increased with time. Although community composition differed between concrete and all other substrates until the completion of my study (Figure 2.5), the limited timescale of these observations (six months) prevents conclusions about potential long-term effects of substrate material. As my study was relatively short-term, it is possible that the effect of material on fouling communities may have diminished or become undetectable after longer periods of submersion.

Experimental plates used in this study were left submerged after the final data collection in mid-November of 2014 with the intent of observing fouling communities the following spring. However, it was discovered that concrete plates had degraded and crumbled away by late April of 2015 (48 weeks post submersion). This breakdown of concrete plates lends support to the hypothesis that chemical leaching from this material may have influenced settlement and growth, perhaps explaining the observed differences in community composition between concrete and all other substrates. However, though concrete plates may have been undergoing micro-scale breakdown processes during the study period, no damage or degradation of experimental substrates was observed before study completion in mid-November 2014.

The findings of this study and others (Vaz-Pinto et al., 2014) suggest that artificial substrates likely do not intrinsically favor non-native species. It is likely that other factors, including substrate orientation, predation exposure, and distance to the water surface, may play a more important role than substrate material itself in explaining the abundance of introduced species on man-made structures. Nevertheless, substrate can influence fouling community composition, and certain materials (e.g. concrete) may provide favorable habitats for particular species (e.g. *C. intestinalis, B. schlosseri*). Extensive usage of certain construction materials might therefore change local-scale composition of fouling assemblages. As these effects seem to be material-specific, targeted studies of the effects of material (including different brands of concrete) on fouling organisms in different geographic locations might inform material usage in marine construction.

Although none of the four materials examined in this study deterred both non-native and cryptogenic (*C. intestinalis*) species, differences in seasonal recruitment patterns may inform coastal management practices and help slow the spread, or limit the dominance of, introduced organisms. Settlement of introduced organisms was greatest from mid-September to mid-October (Figure 2.11). Similarly, though settlement could not be observed in the field, percent cover of the cryptogenic *C. intestinalis* on un-cleaned plates was very low or non-existent before September (Figure 2.7). Conversely, settlement of native organisms peaked from late July to late August (Figure 2.11). Cleaning docks and other submerged structures in July, and avoiding community disturbance in September and October, might allow for greater settlement of native species, and result in less open space for non-native colonization, than other management approaches. If docks are to be removed from the water, dry-land storage of these marine structures by early September would decrease available habitats for non-native organisms during peak recruiting times, possible

leading to subsequent reductions in larval supply. However, studies examining spring recruitment cycles are necessary to determine when to replace these fixtures to maximize native organism settlement and space occupancy.

The findings of this study highlight the importance of settlement plate material choice in experimental marine biology research. As substrate material can influence settlement and community development (McGuinness, 1989; Anderson and Underwood, 1994; Brown, 2005; Andersson et al., 2009; Vaz-Pinto et al., 2014), substrate choice should be tailored to meet experimental goals, or best represent the habitat of interest. Ideally, studies that seek to examine broad scale patterns in benthic ecology should utilize substrates of multiple materials to assure that findings are truly representative. It is also vital that previously completed studies be interpreted with an understanding of how settlement plate material may have influenced experimental findings.

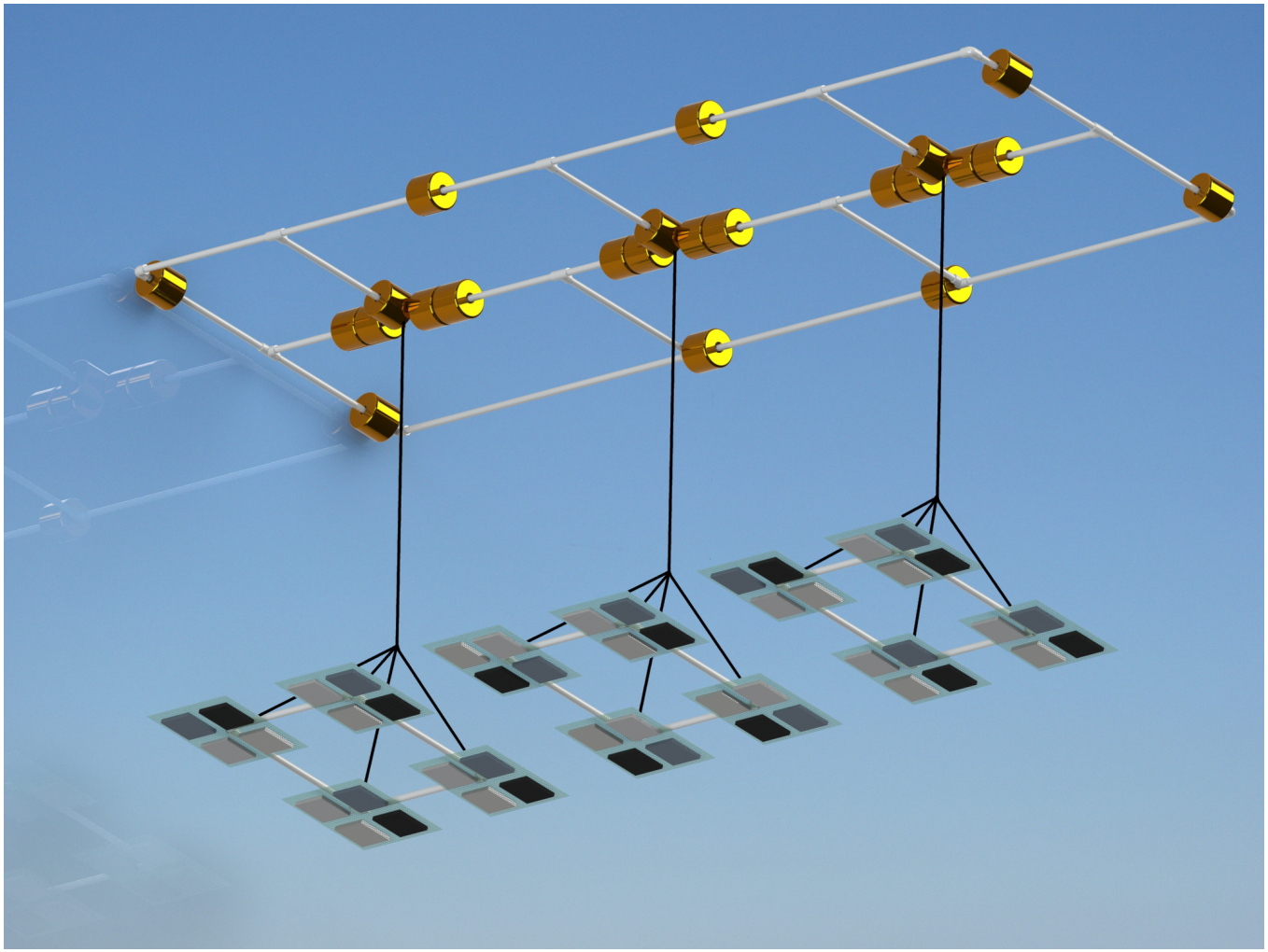

Figure 2.1. Diagram of experimental apparatus as viewed from below, looking up toward the water surface. Settlement plates, composed of concrete, granite, HDPE, and PVC, were randomly arranged within replicate arrays. Image credit: Bart Cromwell.

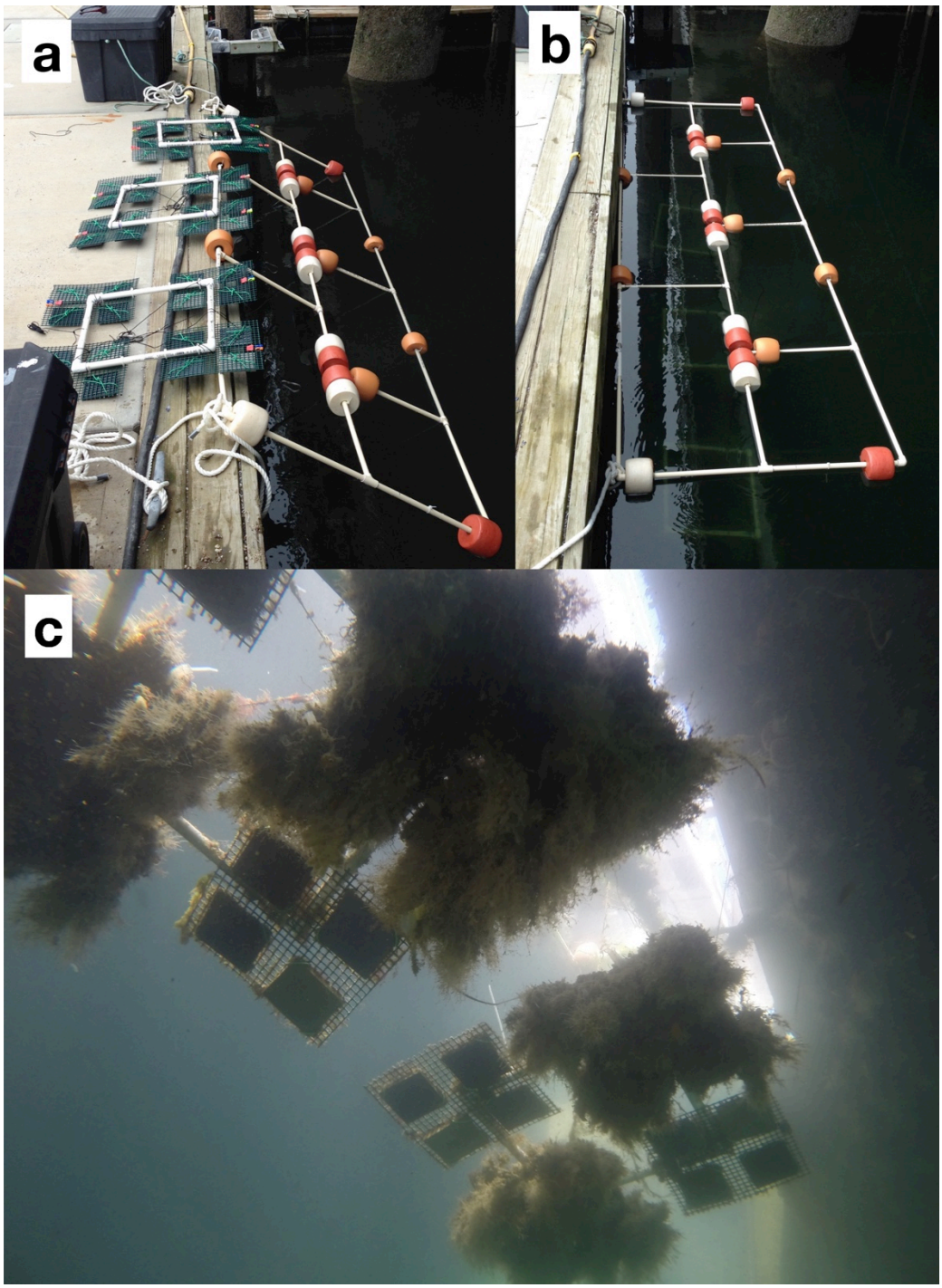

Figure 2.2. Experimental apparatus (a) before and (b) after deployment and (c) viewed from below after 10.5 weeks of submersion. Un-cleaned and bi-weekly cleaned plate arrays are clearly distinguishable.

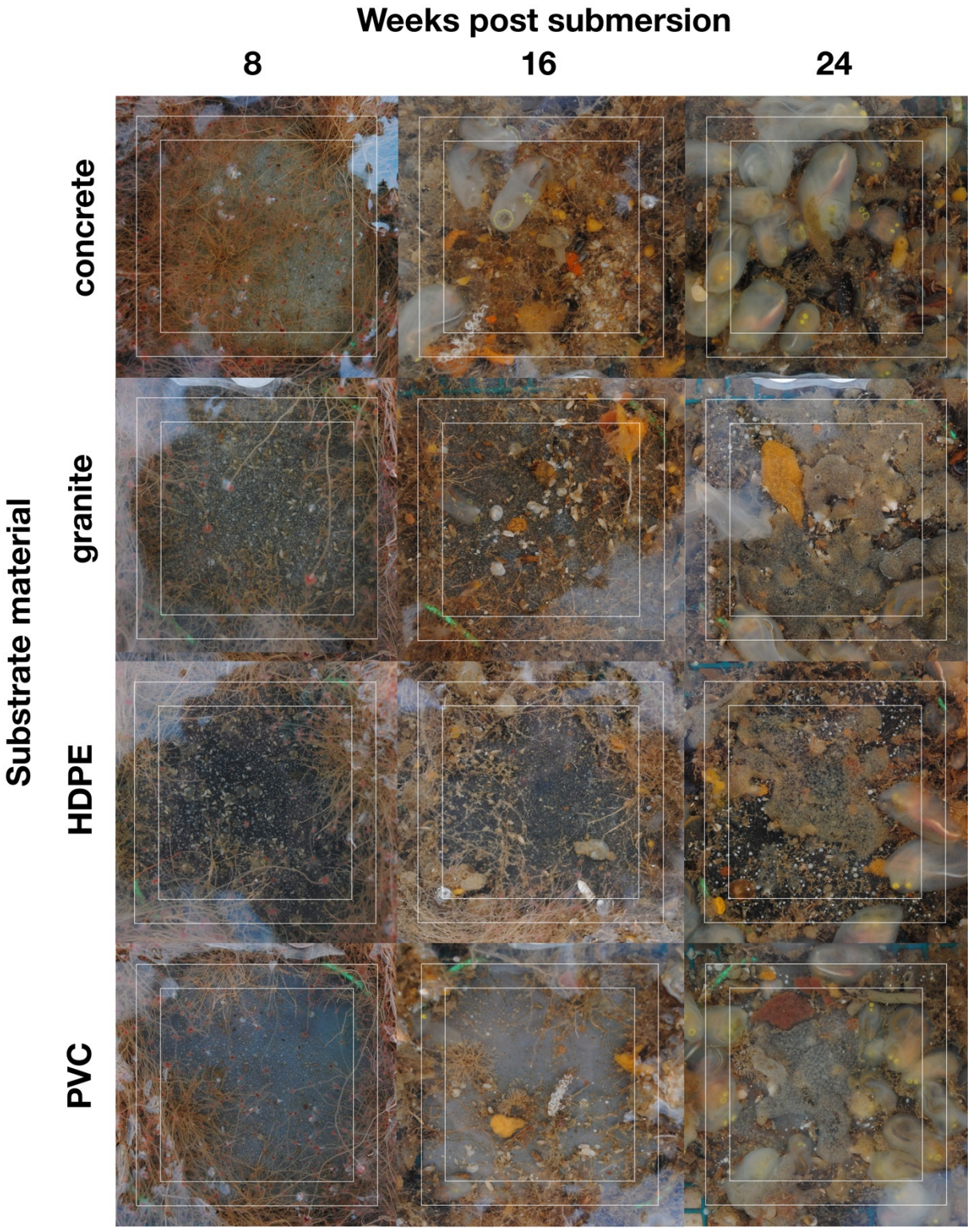

Figure 2.3. Photographs of un-cleaned settlement plates composed of concrete, granite, HDPE, and PVC after 8, 16, and 24 weeks of submersion.

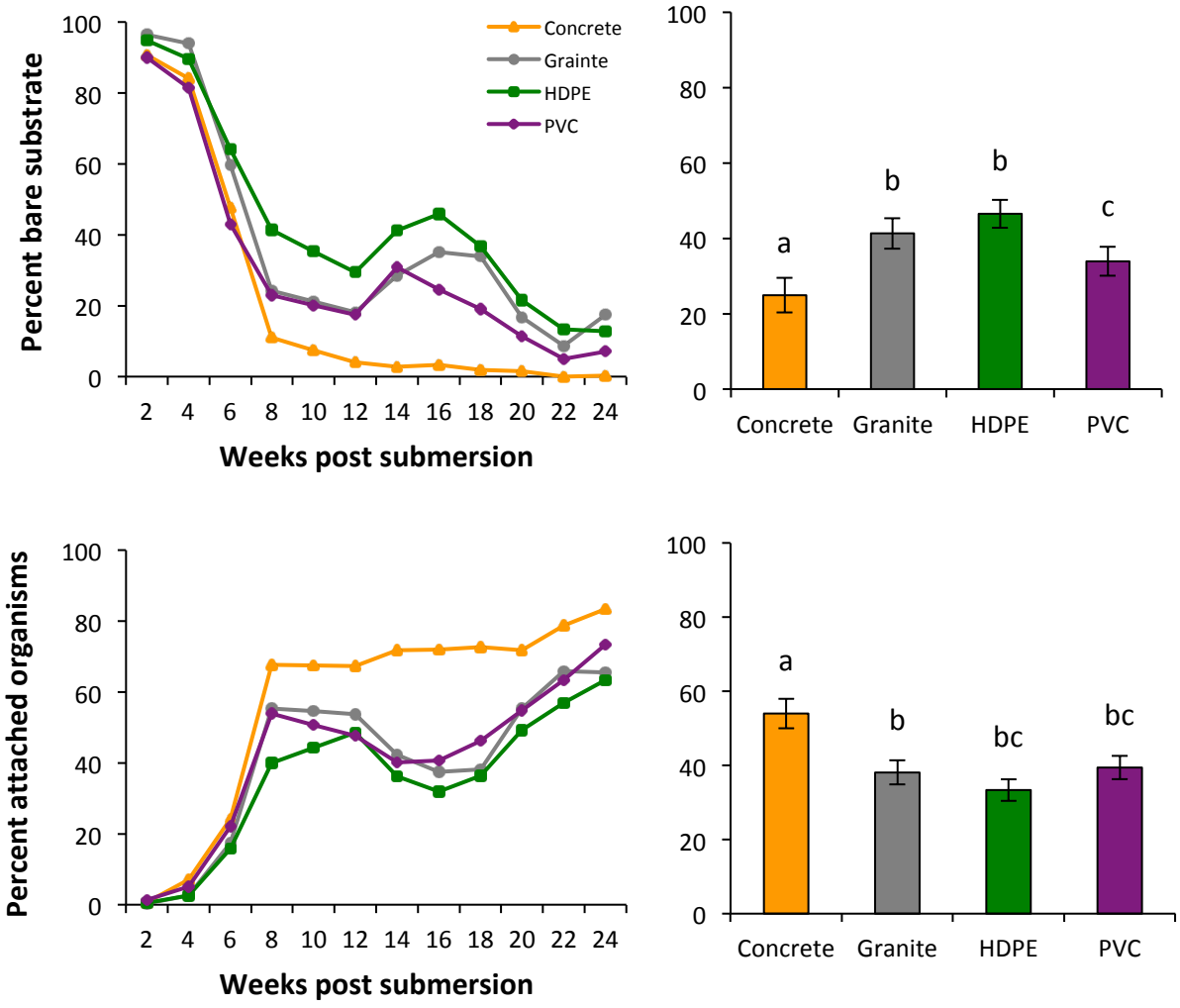

Figure 2.4. Percent cover of bare substrate and all attached organisms on settlement plates composed of four different materials throughout 24 weeks of submersion. Concrete plates had significantly less bare substrate, and greater coverage of organisms, than all other plate materials (see Tables 2.3 and 2.7). N = 6 per material before week 10, N= 5 per material after week 10. Error bars denote ±1 SE.

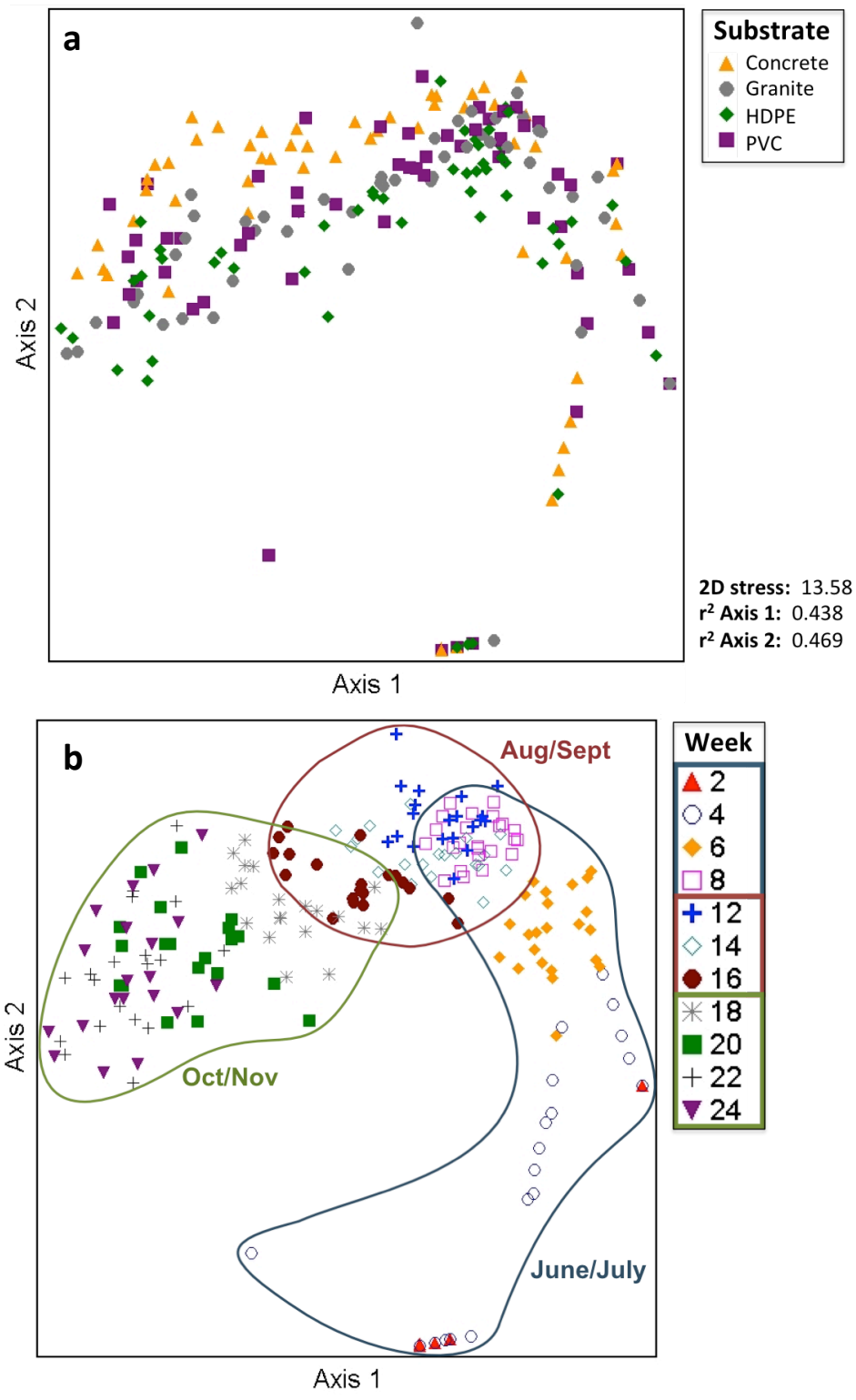

Figure 2.5. Non-metric multidimensional scaling ordination of fouling community composition on un-cleaned plates over 24 weeks of submersion, from May to November. Sample units coded by substrate (a) material or (b) weeks post submersion. (b) Time periods analyzed in subsequent analyses are indicated.

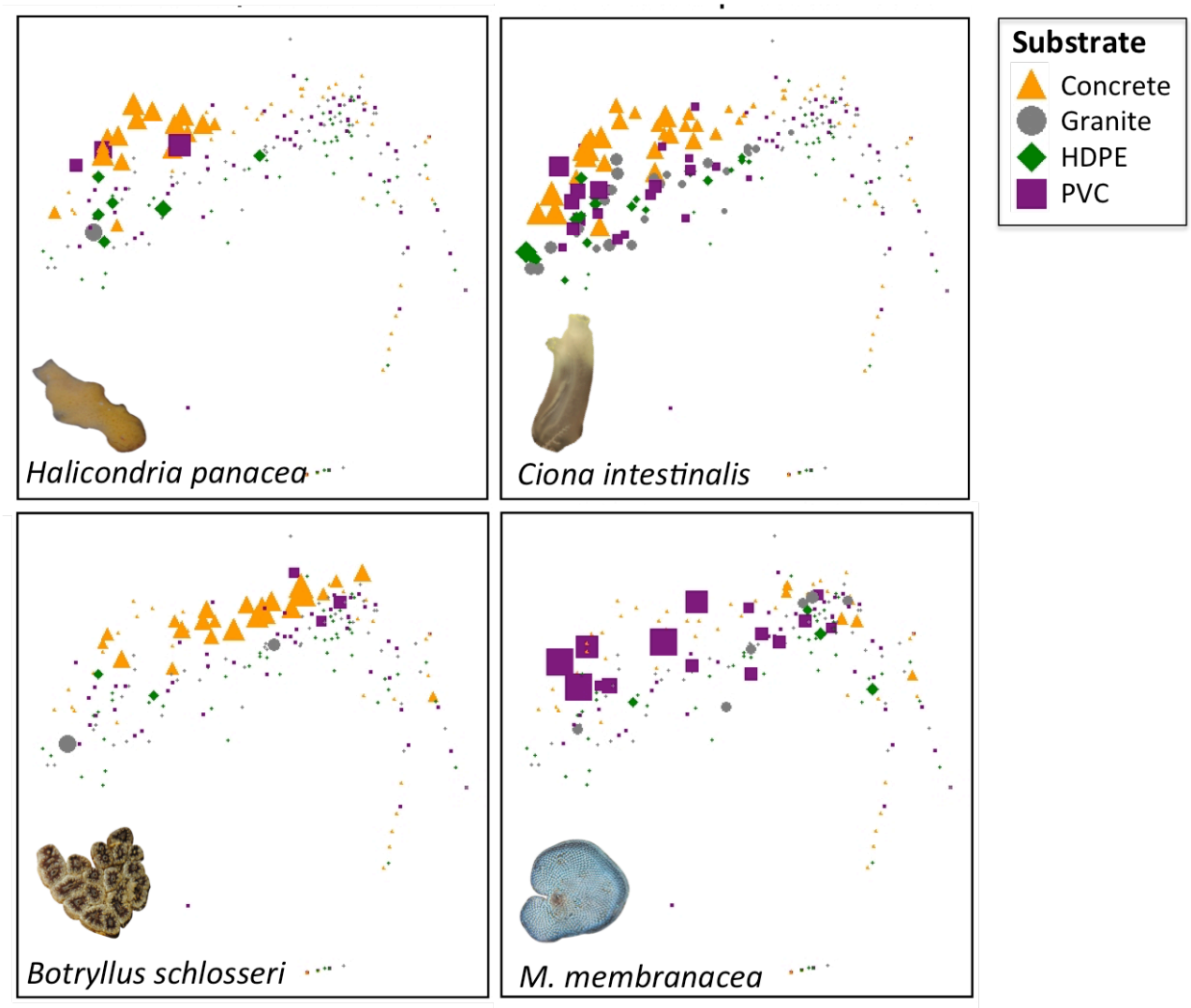

Figure 2.6. NMDS ordinations of community composition on un-cleaned plates with icon size scaled to represent the abundance of four significant indicator species. Each point represents fouling community composition on one settlement plate at one sampling point during the 24-week study. Larger symbols indicate greater percent cover, and smaller symbols indicate lower percent cover, of the species identified in the lower left of each ordination. Note that plates with the highest percent coverage of *Halichondria panicea, Ciona intestinalis,* and *Botryllus schlosseri*, were composed of concrete; these species are all significant indicators of communities on concrete plates. Conversely, coverage of *Membranipora membranacea*, an indicator of PVC, was greatest on PVC plates.

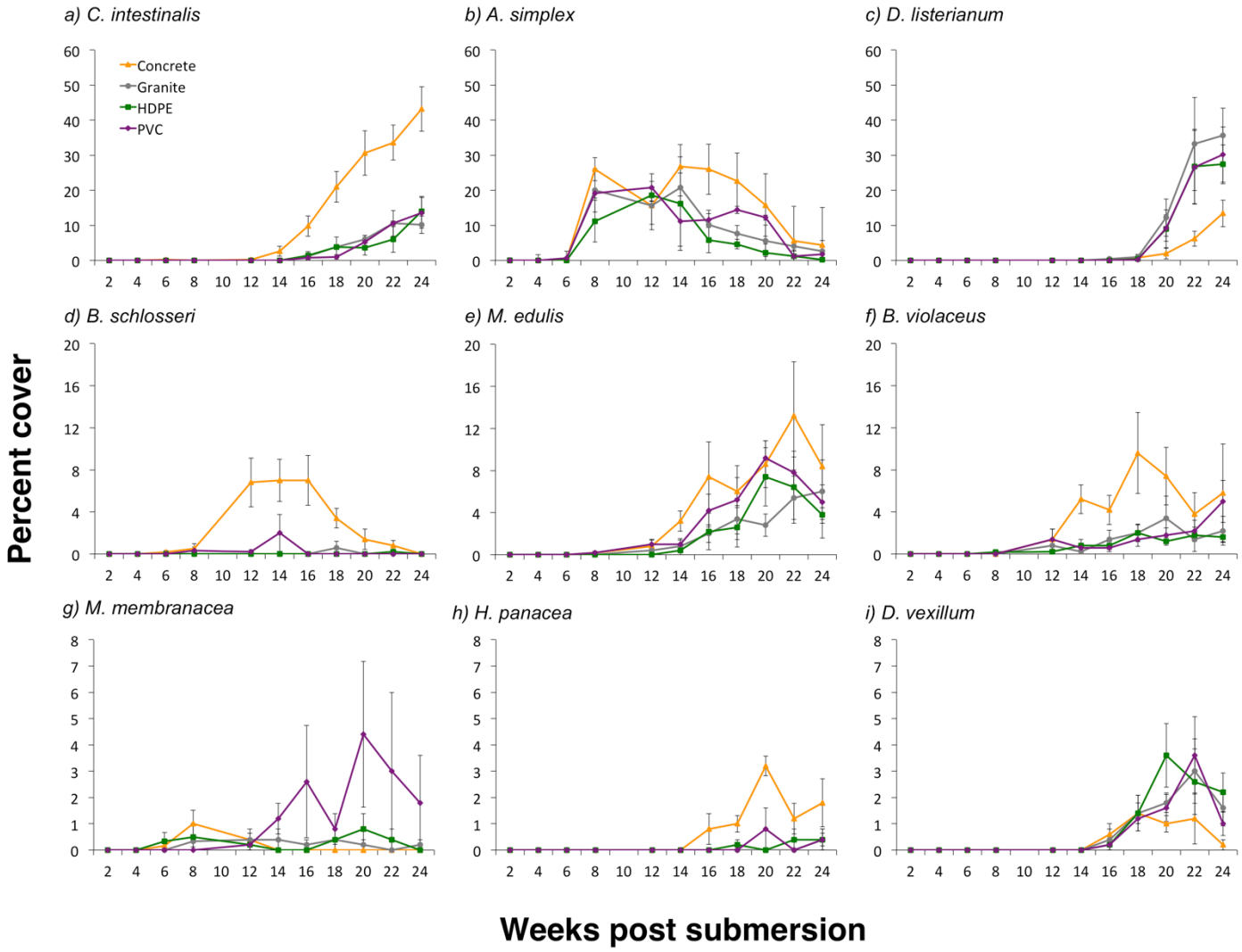

Figure 2.7. Percent cover of nine taxa on un-cleaned settlement plates over 24 weeks of submersion. Note change in y-axis scale between rows. Error bars denote ±1 SE. See Tables 3 and 7 for ANOVA results.

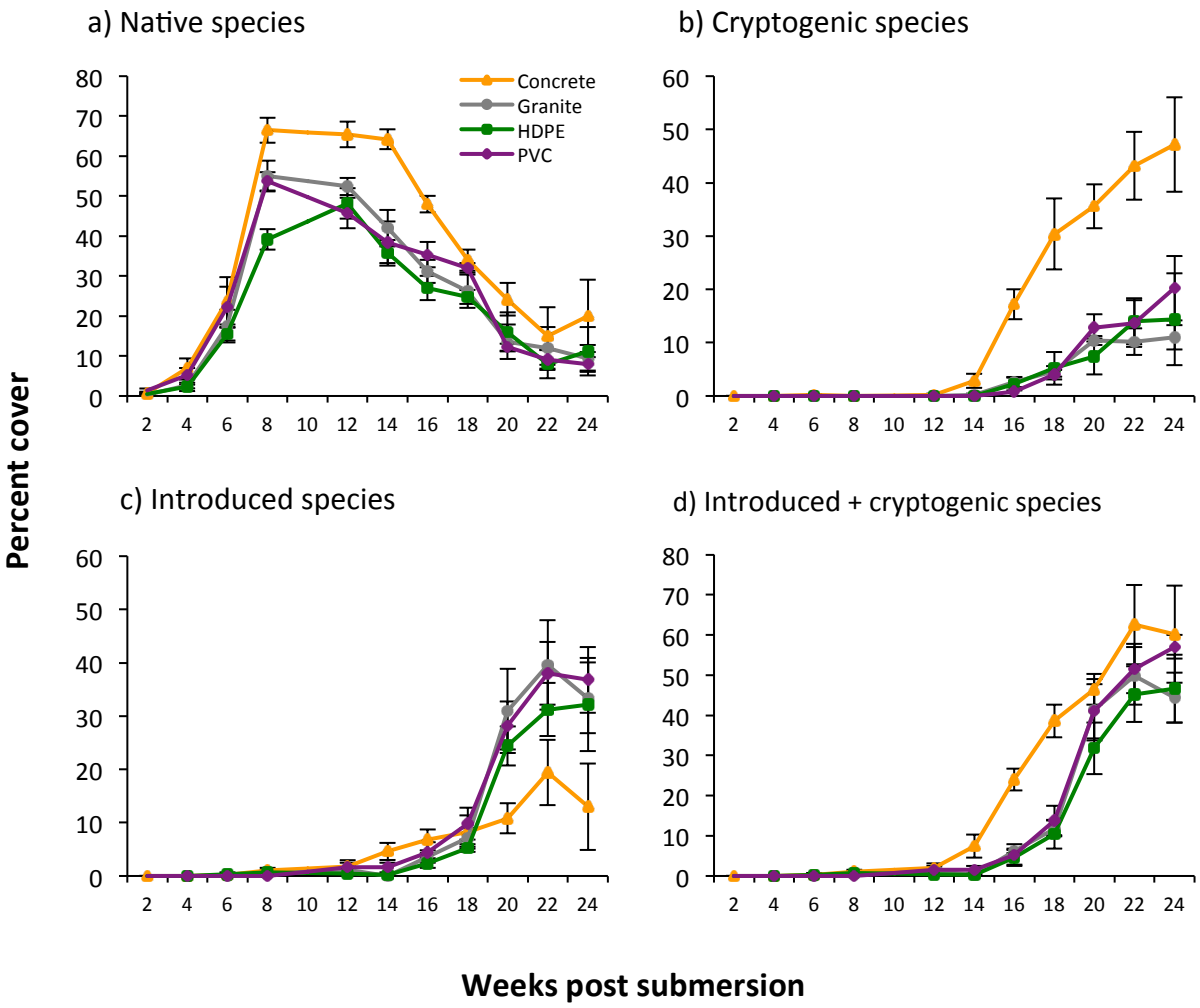

Figure 2.8. Percent cover of native, cryptogenic, introduced, and introduced + cryptogenic organisms on un-cleaned settlement plates throughout 24 weeks of submersion. Note variation in y-axis scale. Error bars denote ±1 SE. See Table 2.1 for species classifications and Tables 2.5 and 2.7 for ANOVA results.

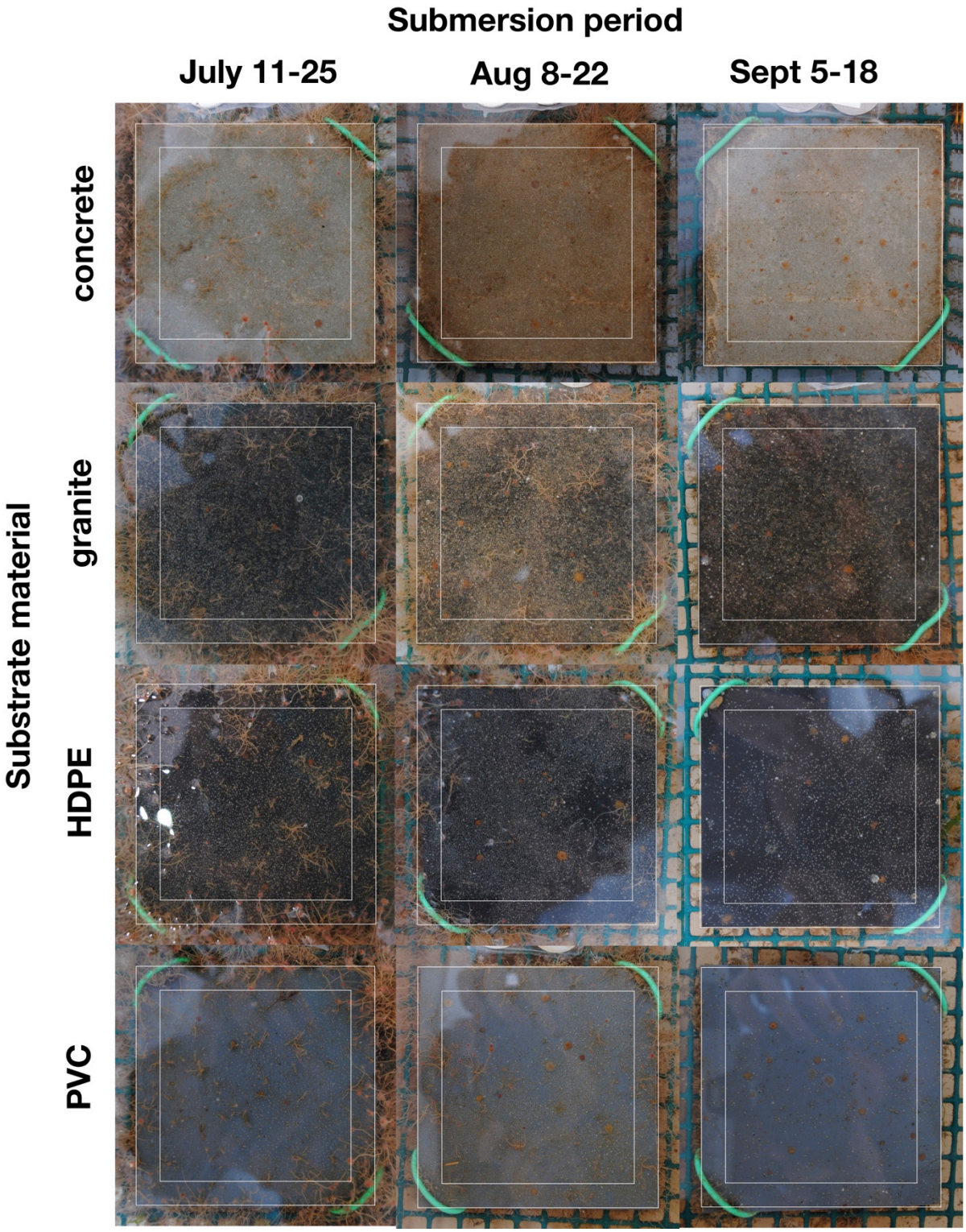

Figure 2.9. Photographs of bi-weekly cleaned settlement plates composed of concrete, granite, HDPE, and PVC submerged from July 11-25, August 8-22, and September 5- 18. Note the low percent cover of organisms, necessitating analysis of communities by organism abundance, rather than percent cover.

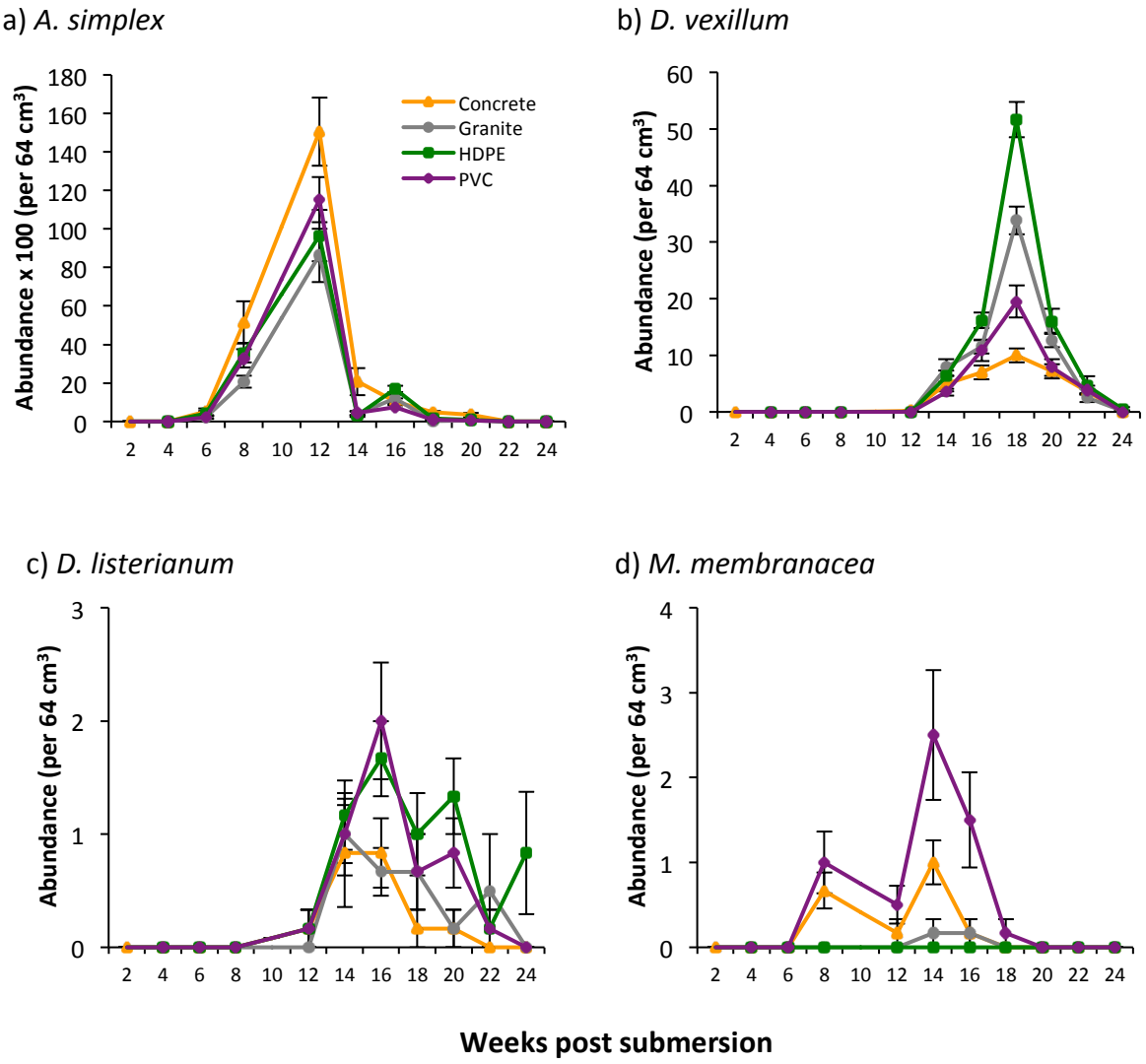

Figure 2.10. Abundance of fouling organism on bi-weekly cleaned settlement plates composed of four different materials during a 24-week study period. Note variation in y-axis scale. Error bars denote ±1 SE. See Tables 2.4 and 2.7 for ANOVA results.

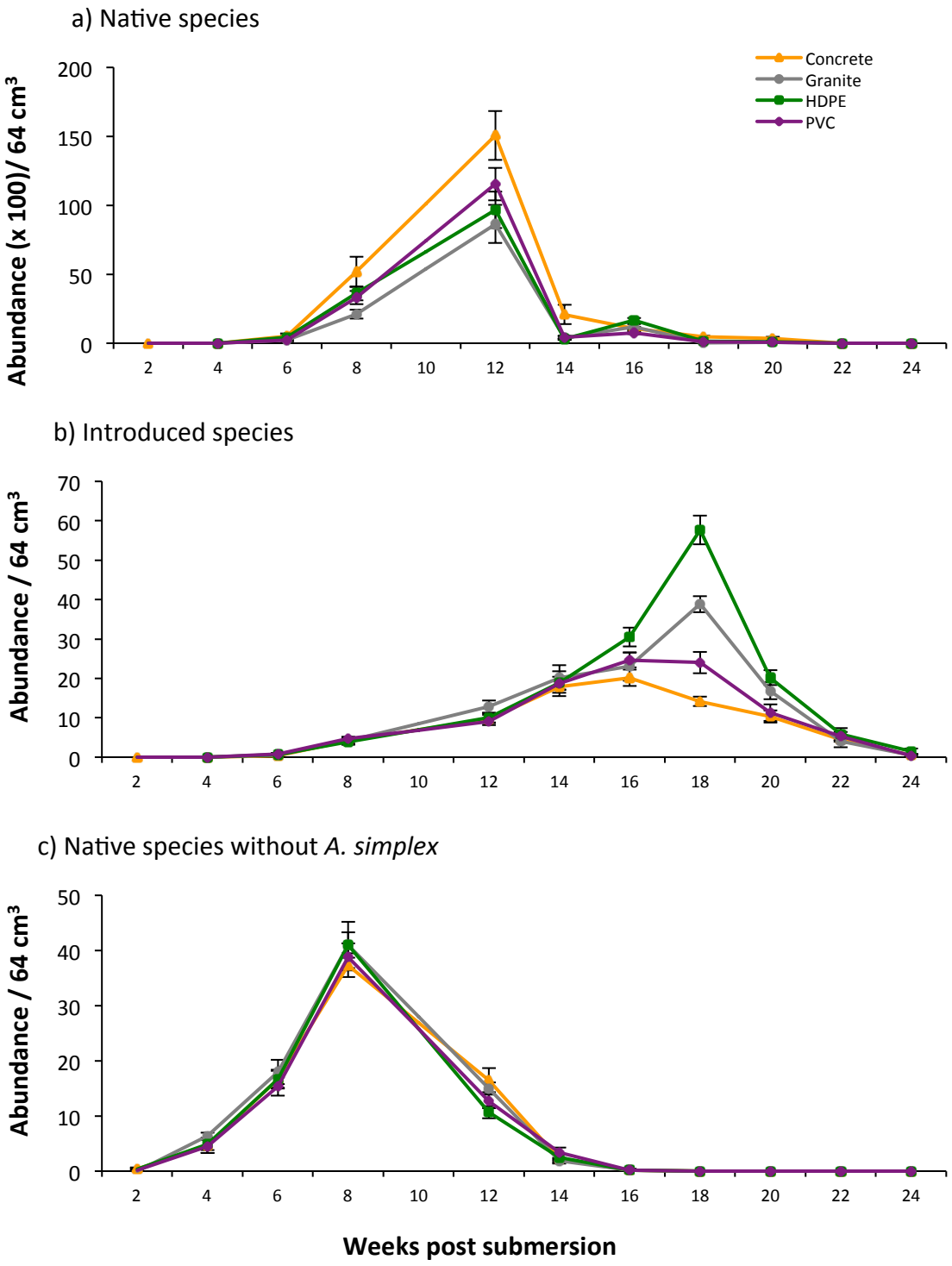

Figure 2.11. Abundance of native species, introduced species, and native species excluding *Anomia simplex* on bi-weekly cleaned settlement plates during the 24-week study period. Error bars denote ±1 SE. See Table 2.1 for species classifications and Tables 2.5 and 2.7 for ANOVA results.

Table 2.1. Classifications of species observed on experimental settlement plates as native (N), cryptogenic (C) or introduced (I) to the Gulf of Maine.

Taxon	Classification	First record in GOM	Source
Anomia simplex	N	-	
Botrylloides violaceus	I	1981	Berman et al. 1992
Botryllus schlosseri	N	before 1870	Gould 1870, Yund et al. 2015
Ciona intestinalis	C	before 1940	Miner, 1950
Didemnum vexillum	I	2001	Bullard et al. 2007
Diplosoma listerianum	I	1993	Harris et al. 1998
Ectopleura crocea	N	-	
Halichondria panacea	N	-	
Hiatella arctica	N	-	
Membranipora membranacea	I	before 1987	Berman et al. 1992, Lambert 1990
Molgula sp.	N	-	
Mytilus edulis	N	-	
Obelia sp.	N	-	

Table 2.2. Indicator Values (IVs) of fouling species associated with substrate material treatments un-cleaned settlement plates in June-July, August-September, and October-November. Only statistically significant indicator species ($p<0.05$) are shown. No indicator species were present on granite substrates.

		June/July		Aug/Sept		Oct/Nov	
Substrate [a]	Species	IV	p value	IV	p value	IV	p value
C	*Anomia simplex*	-	-	31	0.0056	30	0.033
	Botrylloides violaceus	-	-	38	0.0034	-	-
	Botryllus schlosseri	-	-	95	0.0002	19	0.0232
	Ciona intestinalis	-	-	43	0.0006	43	0.0002
	Halichondria panicea	-	-	-	-	50	0.0002
	Mytilus edulis	-	-	32	0.0348	31	0.0402
H	*Didemnum vexillum*	-	-	-	-	30	0.027
	Molgula manhattensis	-	-	-	-	19	0.0454
P	*Membranipora membranacea*	-	-	31	0.0066	33	0.0006

[a] C = concrete, G = granite (not present), H = high-density polyethylene (HDPE), P = polyvinyl chloride (PVC).

Table 2.3. Analysis of variance comparing percent coverage of selected taxa, bare substrate, and all attached organisms, on un-cleaned plates between times (11 data collections over 24 weeks of submersion) and substrate materials (concrete, granite, HDPE, PVC). See Table 2.7 for post-hoc pairwise comparisons of substrate materials. ns: P > 0.05, *P < 0.05; **P < 0.01; ***P < 0.001.

Source	df	MS	F	P	MS	F	P	MS	F	P
		(a) bare substrate			(b) attached organisms			(a) *Botrylloides violaceus*[a]		
Substrate	3	1.337	126.57	***	0.595	45.77	***	0.024	3.416	ns
Time	10	3.136	296.85	***	2.236	171.98	***	0.084	11.739	***
Substrate x Time	30	0.042	3.94	***	0.023	1.757	***	0.007	0.955	ns
Residual	192	0.011			0.013			0.007		
		(b) *Botryllus schlosseri*			(c) *Ciona intestinalis*			(d) Didemnum vexillum		
Substrate	3	0.114	70.622	***	0.474	54.334	***	0.011	6.562	***
Time	10	0.018	11.232	***	0.789	90.506	***	0.064	39.392	***
Substrate x Time	30	0.018	10.954	***	0.046	5.325	***	0.004	2.304	***
Residual	192	0.002			0.009			0.002		
		(e) *Diplosoma listerianum*			(f) *Molgula* sp.			(g) *Ectopleura crocea*		
Substrate	3	0.087	8.542	***	0.013	1.984	ns	0.011	0.894	ns
Time	10	0.947	93.042	***	0.098	14.458	***	1.099	93.626	***
Substrate x Time	30	0.02	1.927	**	0.003	0.407	ns	0.014	1.184	ns
Residual	192	0.01			0.007			0.012		
		(h) *Obelia* sp.[a]			(i) *Halichondria panacea*			(j) *Anomia simplex*[a]		
Substrate	3	0.003	2.288	ns	0.024	18.666	***	0.14	14.166	***
Time	10	0.024	16.477	***	0.01	7.759	***	0.636	64.303	***
Substrate x Time	30	0.003	1.737	ns	0.004	2.976	***	0.017	1.71	ns
Residual	192	0.001			0.001			0.01		
		(k) *Hiatella arctica*			(l) *Mytilus edulis*			(m) *M. membranacea*[a]		
Substrate	3	0.038	6.794	***	0.061	7.406	***	0.033	8.948	***
Time	10	0.051	9.091	***	0.27	33.009	***	0.005	1.392	ns
Substrate x Time	30	0.004	0.762	ns	0.006	0.701	ns	0.006	1.558	ns
Residual	192	0.006			0.008			0.004		

[a] Significance level adjusted to 0.001 due to heterogeneous variances (Levene's test P < 0.05).

Table 2.4. Analysis of variance comparing abundances of fouling species on bi-weekly cleaned plates between times (11 two week submersions over 24 weeks) and substrate materials (concrete, granite, HDPE, PVC). See Table 2.7 for post-hoc pairwise comparisons of substrate materials. ns: P > 0.05, *P < 0.05; **P < 0.01; ***P < 0.001.

Source	df	MS	F	P	MS	F	P	MS	F	P
		(a) *Botrylloides violaceus*			(b) *Didemnum vexillum*[a]			(c) *Diplosoma listerianum*[a]		
Substrate	3	4.187	0.749	ns	410.652	55.986	***	1.904	5.9	**
Time	10	508.09	90.903	***	1888.91	257.525	***	4.887	15.142	***
Substrate x Time	30	3.229	0.578	ns	178.11	24.283	***	0.468	1.45	ns
Residual	220	5.589			7.335			0.323		
		(d) *Ectopleura crocea*			(e) *Anomia simplex*			(f) *M. membranacea*[a]		
Substrate	3	6.111	0.712	ns	1.4E+07	9.869	***	3.677	20.222	***
Time	10	3604.7	419.89	***	2.7E+08	185.279	***	2.136	11.746	***
Substrate x Time	30	6.383	0.744	ns	4880207	3.337	***	2.136	4.929	***
Residual	220	8.585			1462423			0.896		

[a] Significance level adjusted to 0.001 due to heterogeneous variances (Levene's test P < 0.05).

Table 2.5. Analysis of variance comparing percent cover and abundances of native, introduced, and cryptogenic fouling species on un-cleaned, and bi-weekly cleaned settlement plates between times (11 data collections over 24 weeks) and substrate materials (concrete, granite, HDPE, PVC). See Table 2.7 for post-hoc pairwise comparisons of substrate materials. ns: P > 0.05, *P < 0.05; **P < 0.01; ***P < 0.001.

	Uncleaned				Bi-weekly cleaned			
Source	df	MS	F	P	df	MS	F	P
	(a) Native				(e) Native			
Substrate	3	0.863	22.272	***	3	14426551	9.867	***
Time	10	4.42	114.089	***	10	2.72E+08	185.899	***
Substrate x Time	30	0.049	1.268	ns	30	4883171	3.34	***
Residual	192	0.039			220	1462151		
	(b) Cryptogenic							
Substrate	3	0.474	54.334	***				
Time	10	0.789	90.506	***				
Substrate x Time	30	0.046	5.325	***				
Residual	192	0.009						
	(c) Introduced				(f) Introduced			
Substrate	3	0.121	6.619	***	3	461.662	31.296	***
Time	10	1.999	109.6	***	10	3137.411	212.684	***
Substrate x Time	30	0.055	2.996	***	30	196.434	13.316	***
Residual	192	0.018			220	14.752		
	(d) Introduced + cryptogenic				(g) Native without *A. simplex*			
Substrate	3	0.211	8.473	***	3	5.646	0.663	ns
Time	10	5.264	211.501	***	10	3625.115	424.969	***
Substrate x Time	30	0.044	1.785	*	30	6.724	0.788	ns
Residual	192	0.025			220	8.53		

Table 2.6. Analysis of variance comparing univariate community metrics on un-cleaned plates between times (11 data collections over 24 weeks of submersion) and substrate materials (concrete, granite, HDPE, PVC). See Table 2.7 for post-hoc pairwise comparisons of substrate materials. ns: P > 0.05, *P < 0.05; **P < 0.01; ***P < 0.001.

Source	df	MS	F	P	MS	F	P	MS	F	P
		(a) Shannon Diversity			(b) Simpson Diversity			(c) Species Richness		
Substrate	3	0.028	2.012	ns	0.078	1.059	ns	4.684	2.174	ns
Time	9	0.46	33.023	***	2.956	40.206	***	144.294	66.976	***
Substrate x Time	26	0.02	1.464	ns	0.105	1.431	ns	2.511	1.166	ns
Residual	153	0.014			0.074			2.154		

Table 2.7. Post-hoc pairwise comparisons of fouling organism abundance and percent cover, and univariate community metrics, between substrate materials on un-cleaned and bi-weekly cleaned plates. C = concrete, G = granite (not present), H = high-density polyethylene (HDPE), P = polyvinyl chloride (PVC), ns = no significant differences, - = analysis not performed.

	Pairwise comparison results	
Taxon	Uncleaned	Cleaned
Botrylloides violaceus[a]	ns	ns
Botryllus schlosseri	C > G = H = P	-
Ciona intestinalis	C > G = H = P	-
Didemnum vexillum[b]	C < G = H = P	C = P < G < H
Diplosoma listerianum[b]	C < G = H = P	P = H > C = G = P
Molgula sp.	ns	-
Ectopleura crocea	ns	ns
Obelia sp.[a]	ns	-
Halichondria panacea	C > G = H = P	-
Anomia simplex[a]	C > G = H = P	C > G = H = P
Hiatella arctica	G = H > G = C = P	-
Mytilus edulis	C = P > P = G = H	-
Membranipora membranacea[ab]	P > C = G = H	P > C = G = H
Species classifications		
Native	C > G = H = P	C > G = H = P
Native without *A. simplex*	-	ns
Cryptogenic	C > G = H = P	-
Introduced	C = G = H < P = G = H	C = P < G < H
Introduced + cryptogenic	C = P > P = G = H	-
Univariate metrics		
Shannon diversity	ns	-
Simpson diversity	ns	-
Species richness	ns	-

Significance level adjusted to 0.001 due to heterogeneous variances (Levene's test $P < 0.05$) in [a] un-cleaned data, [b] bi-weekly cleaned data.

GENERAL CONCLUSION

Contrary to my hypothesis, settlement of *B. violaceus* and *C. intestinalis* in the field did not align with substrate material preferences exhibited by these species during laboratory experiments (Tables 1.2, 2.4, Figures 1.2, 2.10). *B. violaceus* settled preferentially upon concrete and HDPE plates in the lab (Table 1.2, Figure 1.2), but abundances of this species on bi-weekly cleaned plates did not differ between substrate materials (Tables 2.4, 2.7, Figure 2.10). Although *B. violaceus* was a significant indicator of communities on un-cleaned concrete plates during August and September (Table 2.2), this pattern was not driven by greater larval settlement on concrete plates than on other materials. Unfortunately, it was not possible to observe settlement of *C. intestinalis* on bi-weekly cleaned plates in the field due to the very small size and near transparency of newly settled individuals. However, *C. intestinalis* abundance on un-cleaned plates did not align with expected patterns. In the lab, *C. intestinalis* preferentially settled upon HDPE plates during trials using both 1-3 week and 5-10 week aged plates (Table 1.2, Figure 1.2). In the field, *C. intestinalis* occupied more space on un-cleaned concrete plates than on all other materials, including HDPE (Tables 2.3, 2.7, Figure 2.7). However, as greater settlement onto bi-weekly cleaned plates did not correlate with greater percent coverage on un-cleaned pates for all species, I was unable to determine if *C. intestinalis* exhibited material-specific settlement preferences in the field.

Given the above findings, it seems likely that *B. violaceus* and *C. intestinalis* settlement preferences observed during laboratory studies are not representative of behavior in the field. Numerous differences in physical and biological conditions may explain the discrepancies between

laboratory and field observations. In the lab, larvae were introduced into experimental aquaria within minutes or hours of release from colonies (*B. violaceus*) or hatching (*C. intestinalis*). Because ascidian larvae have short, non-feeding, planktonic stages and rely solely upon stored energy reserves until metamorphosis is complete, this procedure served to assure maximum duration of larval motility within experimental aquaria. Additionally, the design of experimental aquaria assured that larvae were never more than approximately 3cm from an experimental substrate during laboratory experiments. These conditions differ greatly from those in the field. Although adult individuals and colonies of both *C. intestinalis* and *B. violaceus* were located near my field experimental setup on pier pilings and dock surfaces, larvae had to swim or be transported much greater distances in order to contact settlement plates in the field than during lab trials. Therefore, larvae contacting plates in the field were likely older than those in lab studies. Older individuals may be less choosy than newly hatched or released larvae when selecting attachment locations due to their depleted energy stores. Individuals nearing metamorphosis may settle upon any available substrate, and may not exhibit substrate preferences. This may explain why *B. violaceus* settlement on bi-weekly cleaned plates did not differ between substrate materials (Tables 2.7, 2.7, Figure 2.10).

Another factor that may help explain differences in settlement behavior in the lab and the field is plate submersion time, which differed dramatically between studies. Settlement plates were submerged in seawater for 1-3 weeks or 5-10 weeks prior to laboratory trials, whereas settlement was observed in the field over a 24-week period from late May to mid November of 2015. The duration of pre-trial plate submersion was found to influence settlement preferences of *B. violaceus* in the lab (Table 1.2, Figure 1.2), most likely due to changes in chemical leaching rates from different plate materials over time. In the field, *B. violaceus* settlement was first observed

after 6 weeks of submersion, peaked between weeks 12-16 of the study, and continued until 24 weeks after plate deployment. In the lab, *B. violaceus* settled more often than expected by chance only on concrete plates when exposed to 1-3 week aged materials, but during trials using 5-10 week aged plates this species settled preferentially on both concrete and HDPE plates (Table 1.2, Figure 1.2). It is possible that this trend of decreasing material selectivity might increase with submersion time, leading to the observed lack of substrate preference exhibited by *B. violaceus* during the field experiment. However, laboratory studies using plates subjected to longer submersion times are necessary to determine if differences in behavior between lab and field studies resulted from plate aging or other factors.

Although larval age and plate submersion times may help to explain why ascidian behavior differed in laboratory and field settings, it is important to note that observations of organism abundance in the field represented recruitment, rather than settlement, of these species. Settlement, which includes only initial substrate attachment and metamorphosis of larval organisms, was observed during controlled laboratory experiments. However, both substrate attachment and post-settlement survival influenced the abundance of organisms on bi-weekly cleaned plates in the field. Because field plates were only observed once every 14 days, many individuals likely settled upon experimental substrates between data collections but did not persist on experimental substrates long enough to be recorded. Therefore, it is possible that *B. violaceus* may have settled preferentially upon concrete and HDPE plates in the field, but did not survive in greater abundances on these materials, resulting in the observed lack of variation in recruitment. However, it is also possible that post-settlement mortality did not vary on different materials, and recruitment patterns (as observed on bi-weekly cleaned plates) were representative of organism settlement.

These findings highlight the importance of conducting complementary field experiments

when studying larval behavior in a lab setting. Controlled lab studies can provide valuable insight into the larval behavior of species that are difficult to directly observe in the wild (scyphozoa: Holst and Jarms, 2006; ascidians: Rius et al., 2010; but see Stoner, 1994 for direct field observations of *Diplosoma similis* behavior). However, these studies should be regarded as a first step, especially when seeking to answer more applied questions, like how material usage in marine construction might effect organism recruitment or abundance (scyphozoa settlement in both the lab and field: Hoover and Purcell, 2008). For purposes of evaluating these questions, field studies, under real-world conditions, are a necessary complement to laboratory experiments.

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